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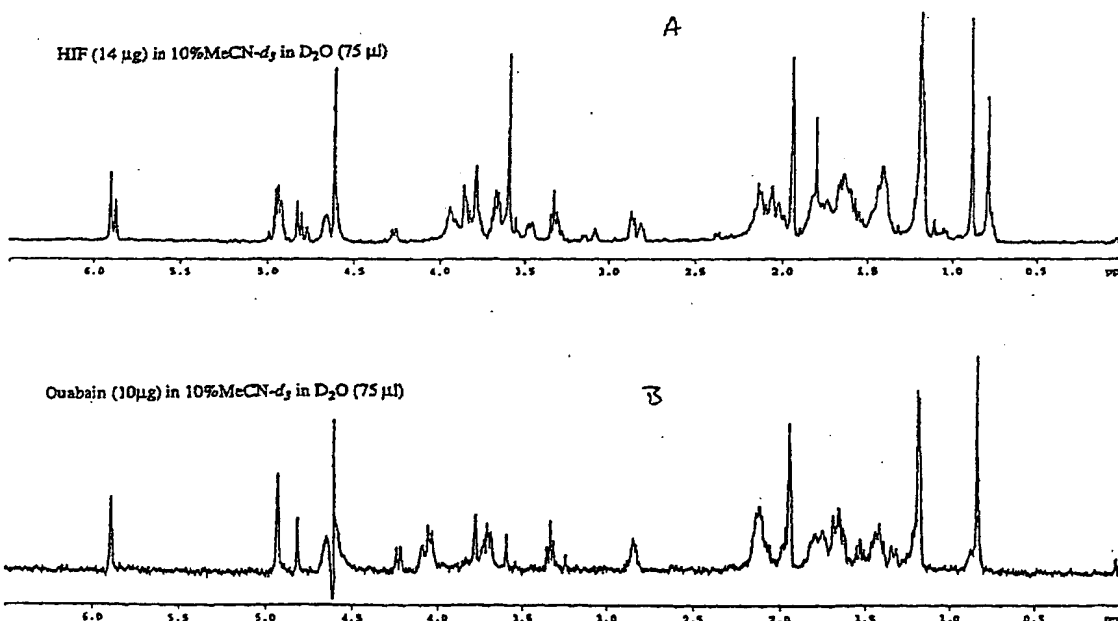
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(57) Abstract

The invention relates to synthetic Na⁺/K⁺-ATPase inhibitory factors, compositions and dosage forms comprising them, and methods of their preparation and use. Specific inhibitory factors of the invention include borate and phosphate complexes of ouabain.

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SYNTHETIC INHIBITORY FACTORS AND METHODS FOR PREPARING SAME

5 This application claims priority to U.S. Provisional Application No. 60/097,325,
filed August 19, 1998, which is incorporated herein by reference.

1. FIELD OF INVENTION

The invention relates to synthetic Na^+/K^+ -ATPase inhibitory factors, compositions comprising them, and methods of their preparation and use.

2. BACKGROUND OF THE INVENTION

10 Despite recent progress in its prevention and treatment, cardiovascular disease remains one of the leading causes of morbidity and mortality in most Western nations. It has long been known that the risk factors for heart disease are numerous and include, for example, family history, elevated serum lipids, cigarette smoking, and hypertension.

15 Although considerable research has been directed at controlling hypertension, the etiology of the disease remains largely unknown (The Merck Manual, 413-415 (16th ed.; 1992)). Both the sympathetic nervous system and the renin-angiotensin-aldosterone system affect blood pressure, but the interaction between the two systems is not entirely clear (see, e.g., Fink, G.D., Clin. Exp. Pharmacol. Physiol. 24:91-5 (1997)). Indeed, the multiple mechanisms controlling renin excretion itself are not completely understood. One of those mechanisms includes a macula densa receptor that appears to detect changes in the delivery rate or concentration of serum sodium chloride. Abnormal sodium transport across cell walls has thus been implicated as one of the factors that affect hypertension.

25 Transmembrane sodium and potassium gradients are maintained by the activity of the sodium pump Na^+/K^+ -ATPase. This enzyme participates in the control of a variety of cellular processes including cellular volume, nutrient transport, calcium content, and cellular excitability (Benaksas, E.J., et al. Prog. Drug Research 45:245-288 (1995)). The activity of the enzyme itself appears, in turn, to be regulated by an endogenous
30 Na^+/K^+ -ATPase inhibitor that is released by the body in response to increased serum sodium chloride levels (see, e.g., Lichtstein, D., et al. J. Basic Clin. Physiol. Pharmacol. 3:269-292 (1992)). Because of the role it appears to play in diseases such as hypertension, considerable research has been directed at identifying this inhibitor.

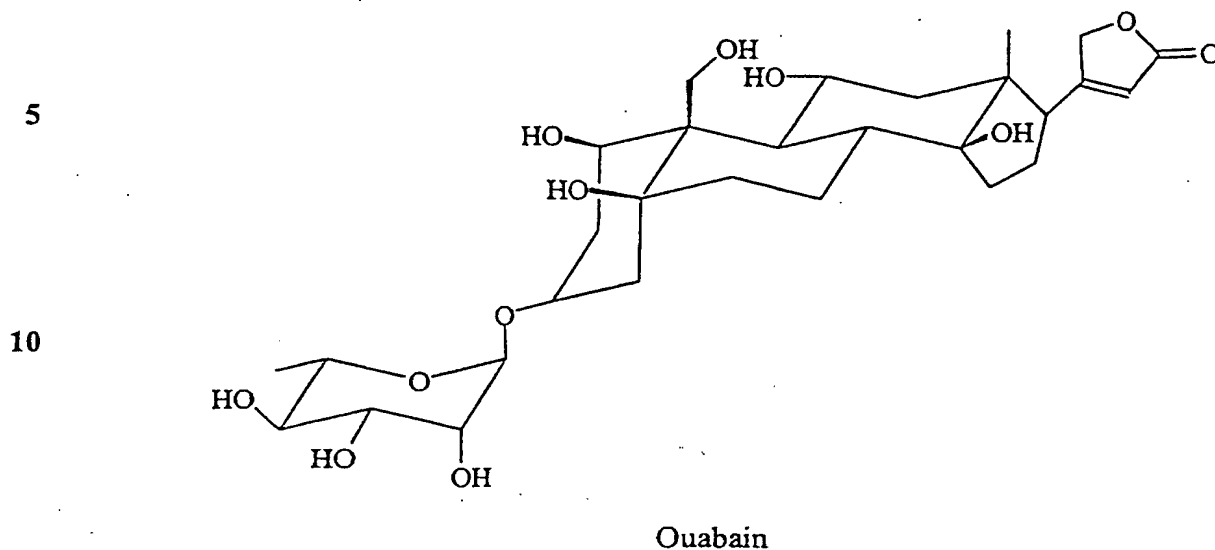
35 A large body of evidence suggests that the endogenous Na^+/K^+ -ATPase inhibitor is structurally similar to cardiotonic factors found in certain toads and plants that have been used to treat congestive heart failure since the late 18th century (Chasalow, F. I., Bradlow, H.L. Ann. N.Y. Acad. Sci. 586:107-116 (1990)). The genin of the plant factors are

polyhydroxylated C₁₇-steroids with an α,β -unsaturated γ -lactone attached to C-17, and various sugars attached to C-3. The genin of the toad factors are C₁₈-steroids to which an α -pyrone is attached at C-17 (Nakanishi, K., et al. *Search for an Endogenous Mammalian Cardiotonic Factor in Saponins Used in Traditional and Modern Medicine* 219-224 (New York: 1996)).

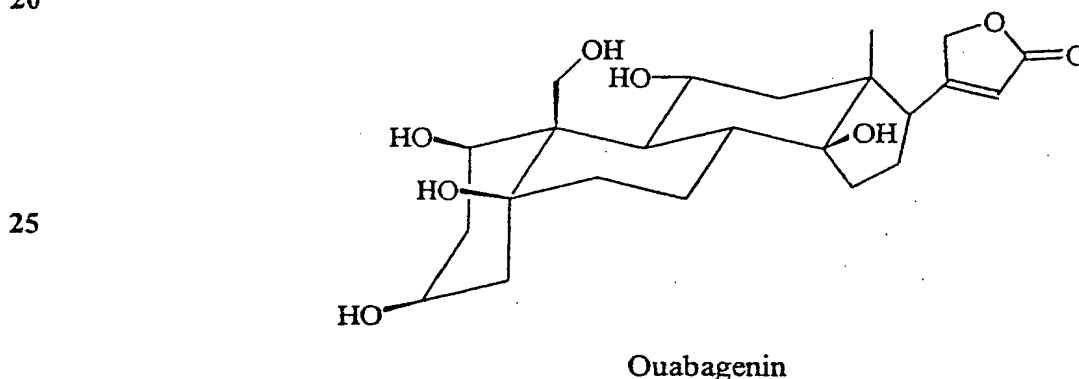
More specific identification of the endogenous Na⁺/K⁺-ATPase inhibitor has been hampered by its very low concentration in mammals and the difficulty in obtaining purified samples for analysis (Tymiak, A.A., et al. Proc. Natl. Acad. Sci. U.S.A. 90:8189-8193 (1993)). Consequently, research has often yielded inconclusive, even contradictory, results (Shaika, I.M., et al. J. Biol. Chem. 266:13672-13678 (1991)). In 1989, however, Hamlyn and coworkers first reported the isolation from human plasma of an ouabain-like compound (OLC) indistinguishable in many respects from the plant glycoside ouabain (Hamlyn, J.M., et al. J. Biol. Chem. 264:7395-7404 (1989); see also Hamlyn, J.M., et al. Proc. Natl. Acad. Sci. U.S.A. 88:6259-6263 (1991)). Similarly, an isomer of ouabain called hypothalamic inhibitory factor (HIF) was isolated from the bovine hypothalamus (Tymiak, A.A., et al. Proc. Natl. Acad. Sci. U.S.A. 90:8189-8193 (1993)). Prior evidence suggested that OLC and HIF were identical, and not ouabain (Zhao, N., et al. Biochemistry 34:9893-9896 (1995)).

The activity of HIF makes it a desirable candidate for the treatment of cardiovascular diseases such as hypertension. However, its use has seemed impractical since it has only been isolated from the bovine hypothalamus in minute quantities and at great expense. For example, studies performed by Hauptert and coworkers were conducted on 3 μ g of HIF collected over a 10 year period from more than 20 kg of bovine hypothalamus (Nakanishi, K., et al. *Search for an Endogenous Mammalian Cardiotonic Factor in Saponins Used in Traditional and Modern Medicine* 219-224 (New York: 1996)).

The isolation, purification and partial characterization of HIF has been reported in the literature (see, e.g., Tymiak, A.A., et al. Proc. Natl. Acad. Sci. U.S.A. 90:8189-8193 (1993); Zhao, N., et al. Biochemistry 34:9893-9896 (1995)), and much work has been directed at determining how it differs from the plant glycoside ouabain:



Similarities between HIF and ouabain have been recognized for some time. For example, glycosidase treatment and acid hydrolysis has provided evidence that, like ouabain, HIF contains the sugar L-rhamnose (Tymiak, A.A., et al. Proc. Natl. Acad. Sci. U.S.A. 90:8189-8193 (1993)). These results suggest that the genin of HIF is ouabagenin:



Although similar in some respects, HIF and ouabain are different compounds. This is clear from their respective nuclear magnetic resonance (NMR) spectra.

FIGS. 1A and 1B show the ^1H NMR spectra of HIF and ouabain, respectively. The spectra differ in at least two respects. First, the ouabain spectrum contains a large singlet at about $\delta=0.85$ ppm attributable to the C-18 methyl group. This is replaced in the HIF spectrum by a pair of resonances at about $\delta=0.78$ and about $\delta=0.87$ ppm. Second, the singlet at about $\delta=5.90$ ppm in the ouabain spectrum attributable to the C-22 olefinic hydrogen is replaced in the HIF spectrum by two peaks centered at about $\delta=5.89$ ppm.

The physiological characteristics or biological activity of natural HIF and ouabain also differ. The Na^+/K^+ -ATPase inhibitory activity of HIF, as measured by the $^{86}\text{Rb}^+$ uptake assay using human erythrocytes, is greater than that of ouabain (Carilli, C.T., et al. J. Biol. Chem. 260:1027-1031 (1985); Anner, B.M., et al. Am. J. Physiol. 258:F144-F153 (1990);
5 Haupt, G.T., et al. Am. J. Physiol. 247:F919-F924 (1984)).

The utility of HIF and related compounds in the treatment of cardiac disorders is clear, despite the fact that HIF has not previously been structurally characterized with certainty. The identification and preparation of such compounds is extraordinarily difficult, however. Consequently, there remains a need for Na^+/K^+ -ATPase inhibitors (including
10 HIF) that are well-characterized, easy to synthesize and have a high affinity for the enzyme's receptor site. These needs are addressed herein.

3. SUMMARY OF THE INVENTION

Using conventional analytical and chemical techniques as well as a combinatorial
15 synthetic approach, it has been discovered that synthetic Na^+/K^+ -ATPase inhibitory factors can be readily prepared from cardenolide compounds. It has further been discovered that some of these compounds have spectroscopic and physiological properties similar to those of HIF and OLC. Preferred cardenolide compounds from which the synthetic factors of the invention can be made include, but are not limited to, ouabain and conformers,
20 stereoisomers, and regioisomers thereof. Synthetic factors of the invention can comprise mixtures or complexes of a variety of cardenolide compounds, but preferred synthetic factors are ethers, ethers, salts, or solvates of ouabain. Most preferred synthetic factors of the invention are borates and phosphates of ouabain.

The invention encompasses synthetic Na^+/K^+ -ATPase inhibitory factors and
25 methods of their preparation. In particular, the invention relates to the preparation of synthetic Na^+/K^+ -ATPase inhibitory factors from cardenolides such as ouabain by the conversion of such as cardenolide to a chemically and biologically distinct inhibitory factor, a mixture of inhibitory factors, or a complex aggregate or mixture of a single compound that has chemical, physical, and biological properties distinct from the purified cardenolide (e.g.,
30 ouabain).

The invention further encompasses a method of preparing a synthetic
 Na^+/K^+ -ATPase inhibitory factor wherein a cardenolide compound such as ouabain is exposed to an atom, compound, or complex capable of forming ionic, hydrogen, or covalent bonds with functional groups bound to cardenolide compound. The exposure is maintained
35 for a time sufficient to obtain the synthetic factor, which can then be isolated.

In a preferred embodiment, ouabain is dissolved in a neutral or basic aqueous solvent and exposed to a source of one or more boron compounds or complexes.

Borosilicate glass has surprisingly been found to be a suitable source of such boron compounds or complexes. In another preferred embodiment, ouabain is dissolved in a solvent and exposed to a source of one or more phosphorous compounds or complexes.

In a second method of preparing synthetic Na^+/K^+ -ATPase inhibitory factors, a steroid or steroid fragment such as ouabagenin is reacted combinatorially with one or more protected derivatives of rhamnose. This reaction yields a mixture from which synthetic inhibitory factors can be isolated and/or purified.

This invention encompasses products produced by any of the methods of the invention, pharmaceutical compositions comprising those products, and their use to treat or prevent disease in a patient (e.g., a mammal, more particularly a human).

3.1 DEFINITIONS

As used herein, the term " Na^+/K^+ -ATPase" refers to the plasma membrane enzyme that catalyzes the active transport of sodium and potassium across cell membranes.

As used herein, the terms "synthetic factor," "synthetic inhibitory factor," "inhibitory factor," and "synthetic Na^+/K^+ -ATPase inhibitory factor" are intended to refer to a compound, a complex, or a mixture of compounds or complexes synthesized by any of the methods of the invention that inhibits the activity of Na^+/K^+ -ATPase, and to all solutions, as well as solvates, polymorphs, salts and prodrugs of said compound, complex or mixture.

As used herein, the term "ouabain borate" refers to a complex, or compound formed by exposing ouabain to a source of atomic, solvated, or complexed boron.

As used herein, the term "ouabain phosphate" refers to a complex or compound formed by exposing ouabain to a source of atomic, solvated, or complexed phosphorous.

As used herein, the term "hypothalamic inhibitory factor" refers to an inhibitor of Na^+/K^+ -ATPase isolated from bovine hypothalamus. See, e.g., Tymiak, A.A., et al. Proc. Natl. Acad. Sci. U.S.A. 90:8189-8193 (1993).

As used herein, the term "ouabain-like compound" refers to a compound comprising a L-rhamnose moiety and having a molecular weight, as determined by ion spray LS/MS, identical to that of ouabain. See, e.g., Hamlyn, J.M., et al. J. Biol. Chem. 264: 7395-7404 (1989).

As used herein, the term "ouabagenin" refers to the genin of ouabain.

As used herein, the term "treatment of cardiovascular disease" means to prevent, reduce, ameliorate or alleviate the symptoms of diseases associated with the cardiovascular system including, but not limited to, arteriosclerosis, atherosclerosis, hypertension, renovascular hypertension, syncope, orthostatic hypotension, shock, heart failure, cardiac arrhythmias, atrial fibrillation, and atrial tachycardia.

As used herein, the term "solvate" refers to any compound or mixture of compounds suspended, mixed, or dissolved in a solvent or solvent mixture.

As used herein, the term "aggregate" means two or more molecules sharing a Van der Waals, ionic, hydrogen-bonding, or similar interaction.

5 As used herein, the term "complex" refers to an aggregate of molecules or atoms, or to molecules or atoms covalently attached to one another. As used herein, the terms "complex" and "compound" are used interchangeably.

As used herein, the term "polymorph" refers a compound or mixture of compounds that has an atomic composition identical to a second compound.

10 As used herein, the term "isomer" includes stereoisomer, regioisomer, polymorph, and conformer of a given compound.

As used herein, the term "pharmaceutically acceptable salts" refers to salts of the synthetic inhibitory factor prepared from pharmaceutically acceptable non-toxic bases, including inorganic and organic bases.

15 As used herein, the term "pharmaceutically acceptable carrier or excipient" refers to a carrier medium or excipient that does not interfere with the effectiveness of the biological activity of an active ingredient, is chemically inert, and is not toxic to the patient to whom it is administered.

As used herein, the term "pharmaceutical compositions" refers to compositions comprising one or more synthetic inhibitory factor, or a pharmaceutically acceptable salt thereof, as an active ingredient. Such compositions can also contain a pharmaceutically acceptable carrier or excipient and other therapeutic ingredients

20 As used herein, the term "protected derivative" means a compound having at least one reactive moiety (e.g., hydroxy) that has been protected. Protecting groups and methods of protecting reactive moieties are well known to those skilled in the art.

3.2 FIGURES

FIG. 1A shows the 300° K, 500 MHz ¹H NMR spectrum of HIF (isolated from bovine hypothalamus) dissolved in 10% acetonitrile-*d*₃ in D₂O.

30 FIG. 1B shows the 300° K, 500 MHz ¹H NMR spectrum of ouabain (Adrich) dissolved in 10% acetonitrile-*d*₃ in D₂O.

FIG. 2A shows the HPLC chromatogram of fractions 7 and 8 of the crude reaction mixture obtained from the combinatorial reaction of ouabagenin and rhamnose as shown in Scheme I.

35 FIG. 2B shows the HPLC chromatogram of fractions 9 and 10 of the crude reaction mixture obtained from the combinatorial reaction of ouabagenin and rhamnose as shown in Scheme I.

FIG. 3 shows the HPLC chromatogram of the reaction mixture obtained by the debenzylation of a mixture comprised of benzylated ouabain and the benzylated 1'-epimer of ouabain.

5 FIG. 4A shows the 300° K, 500 MHz ¹H NMR spectrum of HIF (isolated from bovine hypothalamus) dissolved in 10% acetonitrile-*d*₃ in D₂O.

FIG. 4B shows the 300° K, 500 MHz ¹H NMR spectrum of ouabain (Aldrich) dissolved in 10% acetonitrile-*d*₃ in D₂O.

FIG. 4C shows the 300° K, 500 MHz ¹H NMR spectrum of synthesized ouabain borate dissolved in D₂O.

10 FIG. 5 shows NMR resonance assignments of synthesized ouabain borate in 10% acetonitrile-*d*₃ in D₂O. Chemical shifts are referenced to the methyl groups of acetonitrile-*d*₃ (¹H 1.95 ppm; ¹³C 1.3 ppm).

FIG. 6 shows possible conformers of synthesized ouabain borate.

15 FIG. 7A shows the HPLC chromatogram of naphthoylation product of synthesized ouabain borate.

FIG. 7B shows the HPLC chromatogram of the naphthoylation product of ouabain.

FIG. 7C shows the HPLC chromatogram of the co-injection of the naphthoylation products of ouabain and synthesized ouabain borate.

20 FIG. 8 shows the CD spectrum of the naphthoylation product of synthesized ouabain borate.

FIG. 9 shows mass spectral data for ouabain borate.

FIG. 10 shows the mass spectral fragmentation pattern for synthesized ouabain borate.

25 FIG. 11 shows the relative biological activities of synthesized ouabain borate (indicated by the labels "ak51051," "ak51052," and "ak61502"), HIF (isolated from bovine hypothalamus), and ouabain (Aldrich) in Na⁺/K⁺-ATPase rat α1 isoform assay.

FIG. 12 shows the acidification rate changes of MDCK cells in response to 10⁻⁴ M ouabain. Cells were first stabilized for about an hour, and ouabain was introduced into the cell chamber for 6 minutes.

30 FIG. 13A shows the acidification rate changes in MDCK cells induced by ouabain and ouabain 1,5,19-phosphate. The amounts of the two samples were gradually increased from 10⁻⁸ M to 10⁻⁵ M to see the dose-response relationship. The black thick bars represent the 6-minute periods during which samples were introduced into the cell chamber. The white bar is the background period. Each concentration was applied to the cells for 6
35 minutes, which was followed by 8 minute washing period with the running medium.

FIG. 13B shows the acidification rate changes in MDCK cells induced by 10^{-5} M ouabain and 10^{-5} M ouabain 1,5,19-phosphate. Samples were applied to the cells for 6 minutes (black thick bar).

FIG. 14A shows the acidification rate changes in MDCK cells induced by ouabain and ouabain 1,11,19-phosphate. The amounts of the two samples were gradually increased from 10^{-8} M to 10^{-5} M to see the dose-response relationship. The black thick bars represent the 6-minute periods during which samples were introduced into the cell chamber. The white bar is the background period. Each concentration was applied to the cells for 6 minutes, which was followed by 8 minute washing period with the running medium.

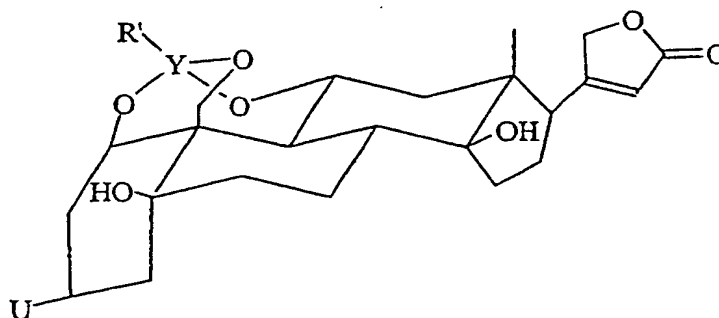
FIG. 14B shows the acidification rate changes in MDCK cells induced by 10^{-5} M ouabain and 10^{-5} M ouabain 1,11,19-phosphate. Samples were applied to the cells for 6 minutes (black thick bar).

4. DETAILED DESCRIPTION OF THE INVENTION

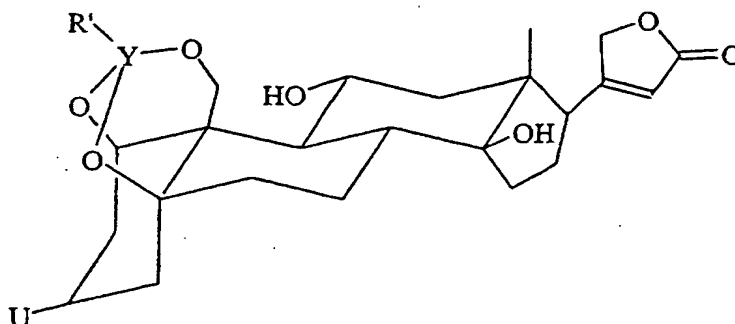
This invention is directed to synthetic Na^+/K^+ -ATPase inhibitory factors and to methods of their preparation and use. Advantageously, compounds of the invention can be prepared from cardenolides such as ouabain. The invention thus represents a significant advance in the art since highly active inhibitory factors distinct from cardenolides such as ouabain can be readily prepared from commercially available materials.

The invention further encompasses pharmaceutical compositions and dosage forms comprising the synthetic Na^+/K^+ -ATPase inhibitory factors disclosed herein. These pharmaceutical compositions and dosage forms can be useful for the treatment and/or prevention of cardiovascular diseases.

A first embodiment of the invention encompasses compounds of Formulas 1 and 2:



Formula 1



Formula 2

and pharmaceutically acceptable salts, solvates, and clathrates thereof and protected derivatives thereof, wherein Y is an atom capable of bonding to two or more of the genin oxygen atoms; R' represents an optional atom or chemical moiety, preferably an atom or chemical moiety that facilitates the formation of Y-O bonds or that contributes to the strength of Y-O bonds under physiological conditions; and U is an atom or chemical moiety such as, but not limited to: hydrogen; hydroxy; alkoxy; ketone; ester, including phosphate ester; carboxylic acid or derivatives thereof; amide; amine; substituted or unsubstituted aryl, alkyl, or aralkyl; halogen; carbohydrate, preferably sugar; or peptide. In specific compounds encompassed by the invention, one of the Y-O bonds is optionally not present, in which case the oxygen atom not bound to Y forms part of a hydroxy or alkoxy group. The invention encompasses racemic and optically pure stereoisomers of compounds of Formulas 1 and 2 as well as crystalline and amorphous forms thereof.

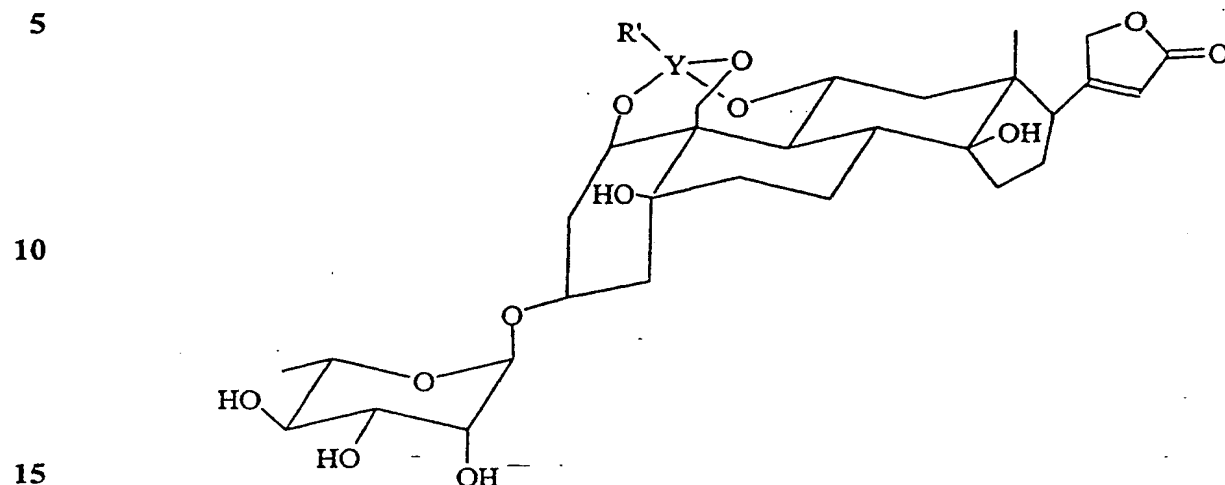
In preferred compounds of the invention, R' is selected from the group consisting of hydrogen, hydroxy, alkoxy, substituted or unsubstituted alkyl, aryl, or aralkyl, oxygen, phosphate or phosphate ester, or the moiety OM, wherein M is a metal (e.g., sodium or potassium). More preferably, R' is oxygen, hydroxy, alkoxy, ester, or a phosphate ester.

In preferred compounds of the invention, U is a moiety of the formula OR, wherein R is hydrogen, substituted or unsubstituted alkyl, aryl, or aralkyl, a metal (e.g., sodium or potassium), or a sugar. More preferably, R is a sugar. Examples of sugars include, but are not limited to, the D and L forms of erythrose, ribose, arabinose, allose, altrose, glucose, mannose, threose, xylose, lyxose, gulose, idose, galactose, talose, erythrulose, ribulose, psicose, fructose, xylulose, sorbose, tagatose, and rhamnose. A preferred sugar is rhamnose.

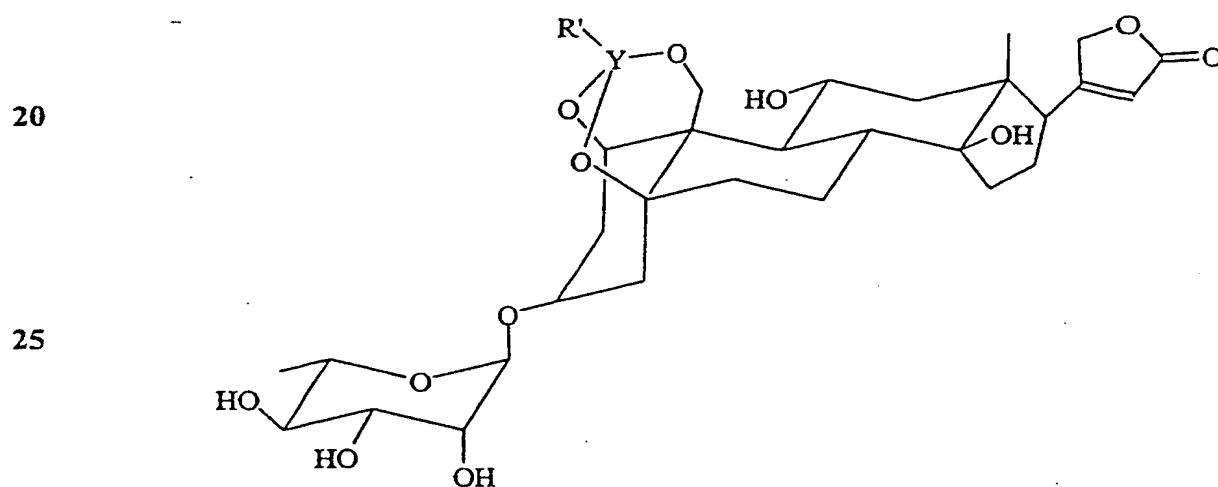
In more preferred compounds of the invention, Y is selected from the group consisting of transition metals (e.g., V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Zr, Nb, Cd, or Sn), boron, carbon, phosphorous, nitrogen, and sulfur. In especially preferred compounds of the

invention, U is OR, R is L-rhamnose, R' is hydroxy or oxygen, and Y is boron or phosphorous.

Most preferred compounds of the invention are those of Formulas 3 and 4:



Formula 3



Formula 4

and pharmaceutically acceptable salts, solvates, and clathrates thereof, wherein Y is boron or phosphorous and R' is oxygen, hydroxy, or alkoxy.

One skilled in the art will recognize that the free hydroxy groups bound to the genin and sugar of the compounds shown above can be substituted or protected, and that such substituted or protected compounds are encompassed by the invention.

Compounds of the invention are stable, solid, and biologically active, and are suitable for use as pharmaceuticals. The phosphate compounds disclosed herein are

particularly stable. The water solubility of many of the compounds of the invention further enhances their utility as pharmaceuticals and aids, for example, in their parenteral administration.

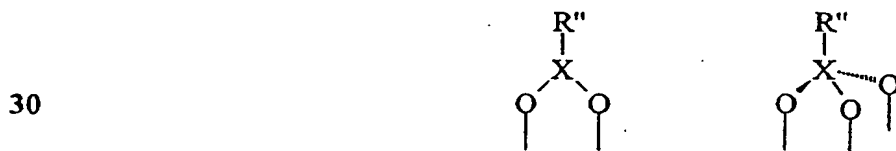
5 4.1 SYNTHESIS OF SYNTHETIC INHIBITORY FACTORS

The Na^+/K^+ -ATPase inhibitory factors of the invention can be synthesized or otherwise prepared from commercially available synthetic material, and can thus be prepared in larger amounts and greater purity than naturally isolated HIF.

10 Inhibitory factors of the invention can be synthesized by a variety of methods, two of which are preferred. In a first preferred method, synthetic inhibitory factor is prepared from a cardenolide compound such as ouabain or an isomer or derivative of ouabain. In a second preferred method, synthetic inhibitory factor is prepared by combinatorial chemistry techniques.

15 In a first embodiment of the invention, synthetic Na^+/K^+ -ATPase inhibitory factor is prepared by exposing a cardenolide compound having at least one functional group to an atom, compound, or complex capable of forming an ionic, hydrogen, or covalent bond with the at least one functional group. The exposure is maintained for a length of time sufficient to allow bond formation. The functional group(s) bound to the cardenolide compound can be, but are not limited to, hydroxy, ketone, carboxylic acid, thiol, amine, amide, and
20 halogen groups. Preferably, the cardenolide compound has at least two such functional groups, and the atom, compound, or complex capable of forming bonds with the functional groups forms bonds with at least two of them.

25 When the cardenolide compound is ouabain or a derivative of ouabain (e.g., ouabagenin) that has hydroxyl groups bound to the steroid skeleton, di- or trietherate complexes can be formed to provide synthetic Na^+/K^+ -ATPase inhibitors of the invention. Such di- and trietherate complexes are represented by Formula 5:



Formula 5

35 wherein X can be, for example, a transition metal (e.g., V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Zr, No, Cd, or Sn), boron, carbon, phosphorous, nitrogen, sulfur, oxygen, magnesium, calcium, or any other element known to those skilled in the art to form di- or trietherate complexes. The optional ligand R' can be any suitable ligand and, like X, can be selected to facilitate formation of the desired cardenolide-etherate complex and to stabilize the complex.

Selection criteria such as valencies and electrophilicity are well known to those skilled in the art. Thus the invention includes, in addition to the preferred borate and phosphate complexes described herein, others such as formate complexes.

5 If cardenolide compounds that contain functional groups other than hydroxy are used to form synthetic inhibitors of the invention, the types of bonds formed will be different, but will be well understood by those skilled in the art. Consequently, the selection of groups necessary for bond formation, such as groups X and R" above, will require little or routine experimentation.

10 The reaction of a cardenolide compound with an atom, compound, or complex capable of forming ionic, hydrogen, or covalent bonds with at least one functional group of the cardenolide compound is preferably enhanced by dissolving the cardenolide compound in a solvent. Selection of an appropriate solvent depends upon the particular solvation characteristics of the cardenolide compound chosen, the nature of its functional groups, and the types of bonds to be formed. For example, when the cardenolide compound is ouabain
15 or an isomer of ouabain, the solvent is preferably water or aqueous alcohol, such as aqueous methanol, ethanol, or propanol, although other solvents known to those skilled in the art can also be used.

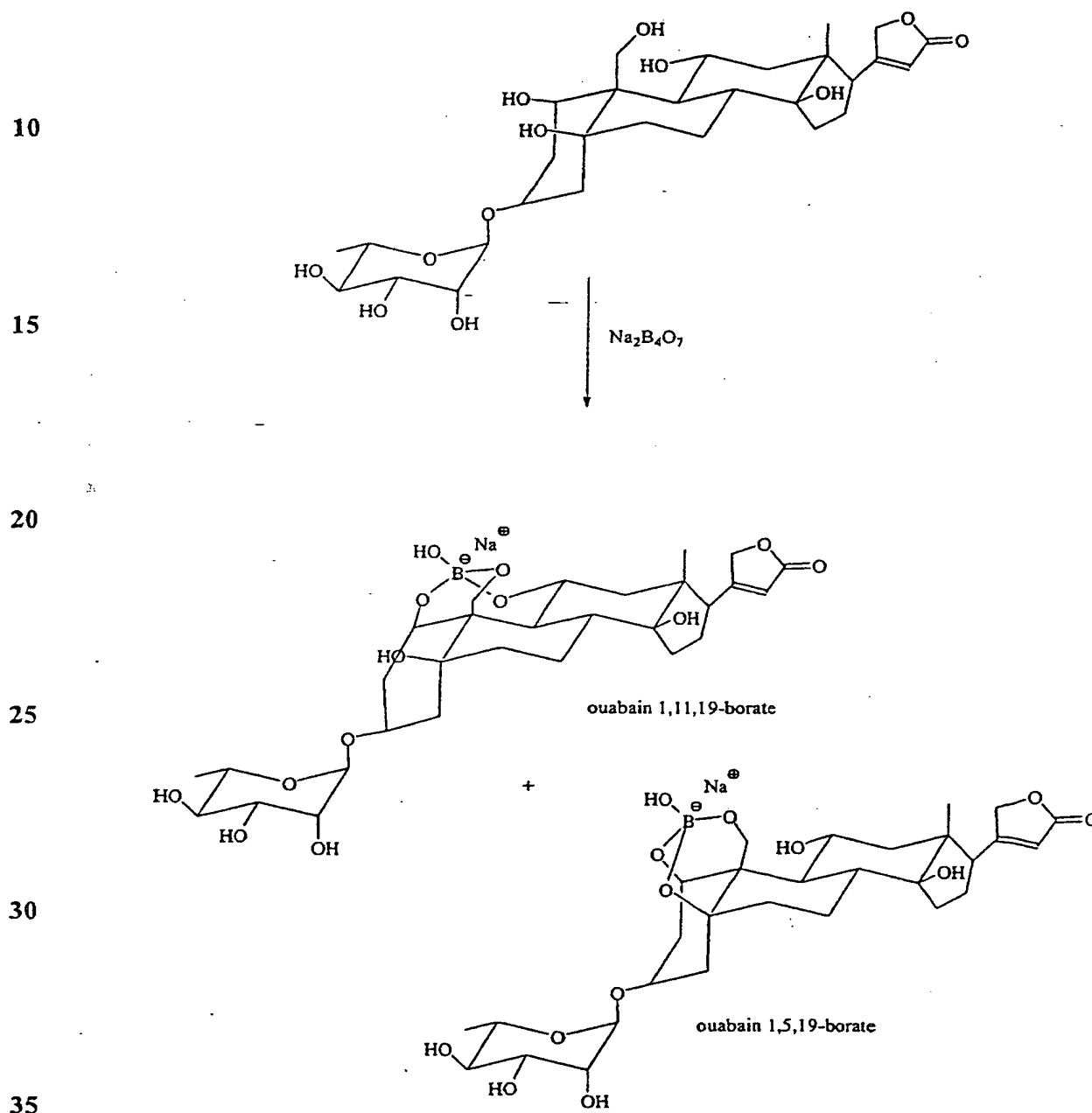
It is commonly known to those skilled in the art that factors such as solvent type, pH, and temperature can affect the bonds formed between certain functional groups and
20 various complexes and compounds. But it is equally well known how such factors should be manipulated in order to facilitate general types of reactions, such as ether or ester formation, as well how they should be manipulated in order to facilitate specific reactions, such as the formation of ethers at certain locations on the steroid skeleton. For example, ouabain borate complexes have been found to form most easily in water at a pH of about 7
25 or higher. The apparent instability of these complexes under acidic conditions is consistent with knowledge possessed by those skilled in the art.

The source of a compound or complex chosen to bond with the functional group(s) of a particular cardenolide compound can be the compound or complex itself, or can be a reactive derivative or salt of the compound or complex. Whatever cardenolide compound is
30 used to form an Na^+/K^+ -ATPase inhibitor of the invention, however, the point at which its complexation with the desired atom, compound, or complex is complete can be determined by spectroscopic techniques such as NMR. Changes in spectral features consistent with those described herein will be readily apparent to those skilled in the art.

4.1.1. PREPARATION OF OUABAIN BORATE COMPLEXES

Ouabain borate complexes are complexes that are formed by the exposure of ouabain to a borate or a source of boron. Without being limited by theory, ouabain borate complexes are believed to be 1,5,19- and/or 1,11,19-borates of ouabain.

5 Ouabain borate complexes are preferably prepared as shown in Scheme 1:



Scheme 1

As shown in Scheme 1, ouabain borate is preferably prepared by reacting ouabain with sodium tetraborate in solution. A wide variety of other boron compounds can also be used to form such complexes, such as, but not limited to, potassium tetraborate and phenylboric acid. Surprisingly, it been found that even borosilicate glass can provide a source of boron suitable for the formation of synthetic inhibitors of the invention.

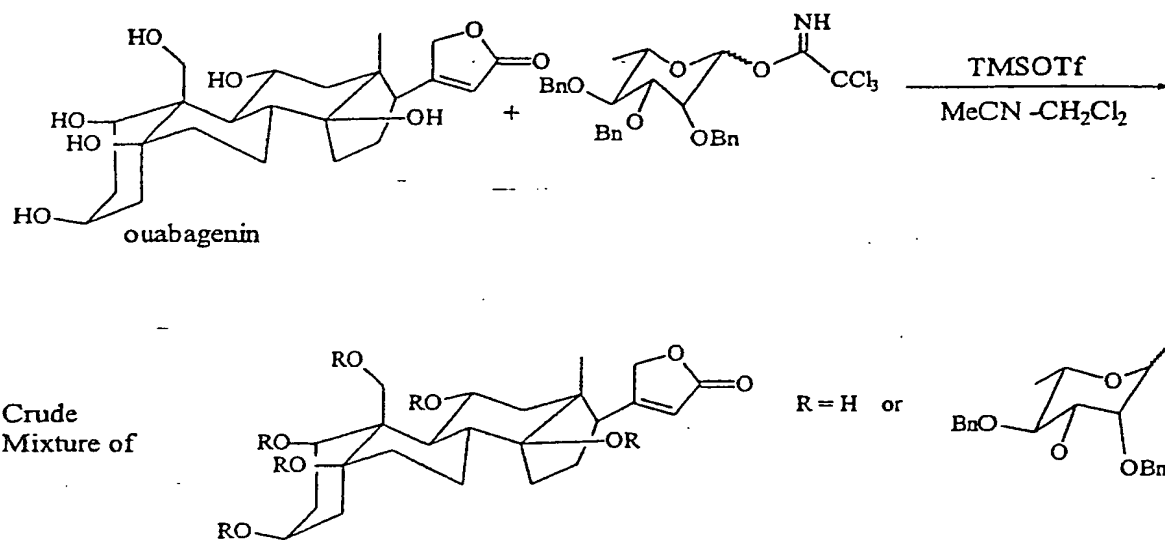
In a second method of preparing an ouabain borate synthetic Na^+/K^+ -ATPase inhibitory factor, a steroid, steroid fragment, or genin, such as ouabagenin with a protected derivative of a sugar such as rhamnose either linearly or combinatorially. This technique has been applied to produce ouabain borate inhibitory factors as shown in Scheme 2:

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Scheme 2

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When the reaction is conducted with ouabagenin and rhamnose, it yields a crude mixture theoretically containing all regioisomers of ouabain, including ouabain itself, and their 1'-epimers (β -isomers at the sugar anomeric position). The components of this mixture can then be separated and purified by conventional techniques if so desired.

Either prior to or after the optional separation and purification of the reaction mixture, a borate or other compound can be added to the reaction products to provide complexes of the invention. It has been found, however, that under neutral or basic conditions, borate complexes will form simply by exposure of the reaction products to borosilicate glassware.

In a variant of this embodiment, protected isomers of ouabain and its 1'-epimer are combined to form a mixture that is subsequently debenzylated to yield the synthetic factor.

4.1.2. PREPARATION OF OUABAIN PHOSPHATE COMPLEXES

Other preferred synthetic Na^+/K^+ -ATPase inhibitors of the invention are ouabain phosphate complexes. Ouabain phosphate complexes are complexes that are formed by the exposure of ouabain to a phosphate or a source of phosphorous. Without being limited by
5 theory, ouabain phosphate complexes are believed to be 1,5,19- and/or 1,11,19-phosphates of ouabain.

Although the combinatorial approach described herein can be used to prepare ouabain phosphate complexes, a preferred methods is shown in Scheme 3:

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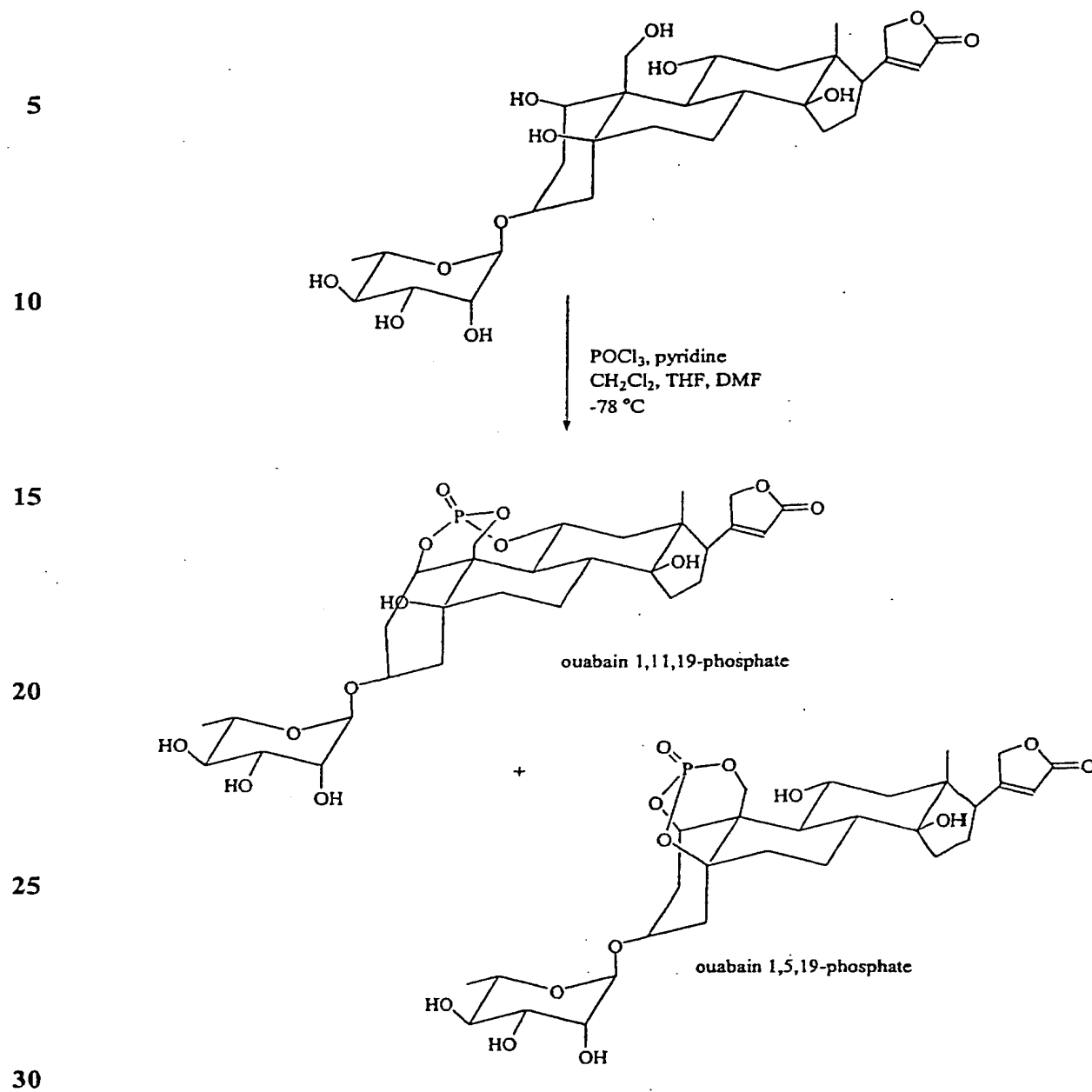
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Scheme 3

According to this method, ouabain 1,5,19- and 1,11,19-phosphates are prepared by treating in a pyridine-containing solvent with phosphorus oxychloride at -78°C. A mixture of the two phosphates (40% yield as a mixture) is typically obtained after silica gel chromatography. Each isomer can then be purified by repetitive reverse phase high performance liquid chromatography (HPLC).

As will be readily apparent to those skilled in the art, phosphate cardenolide complexes other than ouabain phosphate can also be prepared by the method shown in Scheme 3. It will further be apparent that a variety of other methods using different reactants and reaction conditions can also be used to prepare cardenolide phosphate complexes encompassed by the invention.

4.2 PHARMACEUTICAL COMPOSITIONS AND METHOD OF USE

The synthetic inhibitory factors of the invention can be used to treat or prevent diseases in mammals, including humans. Diseases that can be treated include cardiac and cardiovascular diseases such as, but are not limited to, hypertension, angina, edema, hypoxia, arteriosclerosis, atherosclerosis, renovascular hypertension, syncope, orthostatic hypotension, shock, heart failure, cardiac arrhythmias, atrial fibrillation, and atrial tachycardia.

The magnitude of a prophylactic or therapeutic dose of an active ingredient (*i.e.*, a synthetic Na^+/K^+ -ATPase inhibitor) in the acute or chronic management of a disease will vary with the severity of the condition to be treated and its route of administration. The dose and dose frequency will also vary according to the age, weight, condition and response of the individual patient. In general, suitable dose sizes and frequencies of a given synthetic factor can be determined from its biological activity as measured by any of the methods described above.

Any suitable route of administration may be employed for providing the patient with an effective dosage of the active ingredient. For example, oral, rectal, parenteral, transdermal, subcutaneous, intrathecal, intramuscular and the like may be employed as appropriate. Dosage forms include tablets, coated tablets, caplets, capsules, troches, dispersions, sustained release formulations, suspensions, solutions, lyophilized powders that can be reconstituted into solutions, cremes, gels, and patches.

The pharmaceutical compositions of the invention comprise a synthetic Na^+/K^+ -ATPase inhibitor as active ingredient or a pharmaceutically acceptable salt, solvate or clathrate thereof, and may also contain a pharmaceutically acceptable carrier and optionally other therapeutic ingredients.

The pharmaceutical compositions of the invention include compositions suitable for oral, rectal, transdermal, topical, and parenteral administration (including subcutaneous, intrathecal, intramuscular, and intravenous), although the most suitable route in any given case will depend on the nature and severity of the condition being treated. They may be conveniently presented in unit dosage form and prepared by any of the methods well-known in the art of pharmacy.

In practical use, the active ingredient can be combined as the active ingredient in intimate admixture with a pharmaceutical carrier according to conventional pharmaceutical compounding techniques. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, *e.g.*, oral or parenteral (including intravenous
5 injections or infusions). In preparing the compositions for oral dosage form, any of the usual pharmaceutical media may be employed, for example, water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents and the like in the case of oral liquid preparations such as suspensions, elixirs and solutions; or aerosols; or carriers such as
10 starches, sugars, microcrystalline cellulose, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations such as powders, capsules and tablets. Solid oral preparations are generally preferred over liquid ones. The preferred solid oral preparation is tablets. The most preferred solid oral preparation is coated tablets. Because of their ease of administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are
15 obviously employed. If desired, tablets may be coated by standard aqueous or nonaqueous techniques.

Pharmaceutical stabilizers may be used to stabilize compositions comprising a synthetic Na^+/K^+ -ATPase inhibitor. Acceptable stabilizers include, but are not limited to, L-cysteine hydrochloride, glycine hydrochloride, malic acid, sodium metabisulfite, citric
20 acid, tartaric acid, and L-cystine dihydrochloride. *See, e.g.*, U.S. Patent Nos.: 5,731,000; 5,763,493; 5,541,231; and 5,358,970, all of which are incorporated herein by reference.

In addition to the common dosage forms set out above, the active ingredient is preferably administered by controlled release means and/or delivery devices capable of releasing the active ingredient at a rate required to maintain constant pharmacological
25 activity for a desirable period of time. Such dosage forms provide a supply of a drug to the body during a predetermined period of time and thus maintain drug levels in the therapeutic range for longer periods of time than conventional non-controlled formulations. Examples of controlled release pharmaceutical compositions and delivery devices that may be adapted for the administration of the active ingredients of the present invention are described in U.S.
30 Patent Nos.: 3,847,770; 3,916,899; 3,536,809; 3,598,123; 3,630,200; 4,008,719; 4,687,610; 4,769,027; 5,674,533; 5,059,595; 5,591,767; 5,120,548 ; 5,073,543; 5,639,476; 5,354,566; and 5,733,566, the disclosures of which are incorporated herein by reference. Preferred controlled release means are disclosed by: U.S. Patent Nos. 5,427,798 and 5,486,362; WO 9404138; CA 1239034; and European Patent Application Nos. 467488 and 1 71457, all of
35 which are incorporated herein by reference.

Pharmaceutical compositions of the invention suitable for oral administration can be presented as discrete units such as capsules, cachets, or tablets or aerosol sprays, each

containing a predetermined amount of the active ingredient as a powder, as granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, an oil-in-water emulsion, or a water-in-oil liquid emulsion. Such compositions may be prepared by any of the methods of pharmacy which include the step of bringing into association the active ingredient with a carrier which constitutes one or more necessary ingredients. In general, the compositions are prepared by uniformly and intimately admixing the active ingredient with a liquid carrier or a finely divided solid carrier, or both, and then, if necessary, shaping the product into the desired presentation. For example, a tablet may be prepared by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as powder or granules, optionally mixed with a binder, lubricant, inert diluent, and/or surface active or dispersing agent. Molded tablets can be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

Further benefits provided by this invention will be apparent from the following

Examples.

5. EXAMPLES

Unless otherwise noted, the following materials were used to prepare and analyze the synthetic inhibitors described in the Examples. Ouabain octahydrate and sodium tetraborate decahydrate were obtained from ACROS, Hampton, NH, while all other chemicals, HPLC solvents, and dog kidney Na^+, K^+ -ATPase ($\alpha 1$ isoform) were obtained from Aldrich, Milwaukee, WI. MDCK cells were obtained from ATCC. Penicillin-Streptomycin, Fungizone[®], 2.5% trypsin, Hanks' balanced salt solution (HBSS) and Dulbecco's modified-Eagle medium (D-MEM) were purchased from Life Technologies, Rockville, MD. Fetal bovine serum (BioWittaker), culture flasks, pipets, vials, were obtained from Fisher Scientific, Pittsburgh, PA. Extracellular acidification rate was measured with Cytosensor[™] (Molecular Devices, Sunnyvale, CA), and the obtained data were analyzed with Cytosoft[®] Version 2.0.1. Capsule kit, filter membrane, cleaning solutions for Cytosensor[™] were purchased from Molecular Devices. NMR spectra were recorded on a Bruker DMX500 (¹H, COSY, HMQC, HMBC, ROESY) or a Bruker DRX300WB (¹³C, DEPT, ¹¹B) spectrometer. NMR samples were typically dissolved in 10% acetonitrile-*d*₃ in deuterium oxide. The methyl group of acetonitrile-*d*₃ or methanol-*d*₃ was used as an internal standard for the determination of ¹H- and ¹³C-NMR chemical shifts. 85% H₃PO₄ was used as an external standard for the determination of ³¹P-NMR chemical shifts.

5.1. SYNTHESIS OF OUABAIN BORATE COMPLEX

The following is intended to illustrate the preparation of ouabain borate. It should be readily apparent to those skilled in the art that the following, and variations of the following, can be used to prepare any of a variety of ouabagenin or cardenolide derivatives suitable for use as Na⁺/K⁺-ATPase inhibitors.

5.1.1. CONVERSION OF CARDINOLIDE COMPOUNDS

Approximately 10 µg ouabain octahydrate (ACROS) was dissolved in 100 µl of water and placed in a 1 dram borosilicate vial (Kimble Opticlear®), where it was kept at room temperature overnight. Lyophilization of the resulting material yielded a white powder comprising the inhibitory factor of the invention.

Although it has been found that this method of forming ouabain borate works with amounts of ouabain octahydrate as large as 100 µg, it appears to be more efficient when smaller amounts are used.

A larger amount of ouabain borate was obtained by dissolving ouabain octahydrate (50 mg; 68.6 µmol) in approximately 10 mL of water. To this solution were then added sodium tetraborate (6.5 mg, 17.2 µmol) and 2.5 mL of 14 mM sodium hydroxide. The resulting mixture was stirred at room temperature for approximately one hour, and then lyophilized to yield a white powder comprising ouabain borate.

It has also been found that simply mixing ouabain with an excess amount of sodium tetraborate without the addition of base gives a borate complex of the invention.

5.1.2. COMBINATORIAL SYNTHESIS

In approximately 30 mL acetonitrile/methylene chloride (2:1 v/v), 100 mg benzylated L-rhamnose (SIGMA, St. Louis, MO) was combined with 130 mg ouabagenin (SIGMA). After the mixture was cooled to -10°C, 500 µL of TMSOTf (10% solution in methylene chloride) was added.

Upon completion of the reaction, the reaction mixture was loaded on a 1x15 cm silica gel open column with 1% methanol in methylene chloride. The components of the mixture were then eluted with 3% methanol in methylene chloride (3 columns) followed by 5% methanol in methylene chloride (5 columns). Twelve fractions were isolated. As shown in FIG. 2, fractions 7-10 had the same R_f value as measured by silica gel thin layer chromatography (10% methanol in methylene chloride) the R_f value of fractions 7-10 in methanol/CHCl₃ (1:9) was 0.45. TLC plate size was 3x5 cm.

Identification of the contents of fractions 7-10 involved their purification by HPLC (Waters NovaPak cartridge (7.8 x 300 mm); monitoring absorption at 220 nm) using the elution conditions provided in Table 1.

Table 1. HPLC Elution Conditions

Eluent	Time (min) (3 mL/min)			
	0	10	15	20
5% MeCN (aq)	50%	50%	0%	50%
100% MeCN	50%	50%	100%	50%

In general, however, isolation of this synthetic inhibitory factor of this invention does not require purification of the fractions by HPLC.

Fractions 7 and 8, the earlier fractions of the $R_f=0.45$ product, were combined and analyzed by reverse phase HPLC (Waters Nova Pak C_{18} , 7.8 x 300 mm) to provide the chromatogram shown in FIG. 2A. Fractions 9 and 10, the later fractions of the $R_f=0.45$ product, were also combined and analyzed by reverse phase HPLC. The combined fraction provided the chromatogram shown in FIG. 2B. FIGS. 2A and B show that the earlier (7 and 8) and the later (9 and 10) fractions contain the same components in different ratios.

The earlier (7 and 8) and later (9 and 10) fractions were separately debenzylated (10% Pd/C, H_2 , in methanol/ethyl acetate (3:1)). One of the debenzylated products from earlier fractions had a HPLC retention time same as ouabain. Products from fractions 9 and 10 also contained a compound with the ouabain retention time. However, while both the earlier (7 and 8) and later (9 and 10) fractions gave products with a retention time equal to that of ouabain's, 1H -NMR of these products indicated they are in fact different.

The 1H NMR spectrum of the purified combination product obtained from fractions 7 and 8 is similar to that of ouabain, but contains some weak resonances characteristic of HIF. The spectrum of the combination product obtained from fractions 9 and 10 indicates that the boron present in the glassware used can be sufficient to form borate complexes of the invention.

5.1.3. COMBINATORIAL SYNTHESIS

As above, benzylated L-rhamnose was reacted with ouabagenin to yield a mixture of products that was subsequently run down a silica gel open column. Twelve fractions were isolated and purified by HPLC to yield the purified benzylated precursors of ouabain and its 1'-epimer. These two compounds were then combined in a ratio of about 1:3 (ouabain/1'-epimer) to form a mixture that was subsequently debenzylated. The components of the reaction mixture were separated by reverse-phase HPLC (Waters NovaPak, 7.8 x 300 mm; eluted over 20 minutes with an acetonitrile/water (10% to 15% acetonitrile) gradient at 3 mL/min; monitoring absorption at 220 nm).

FIG. 3 shows the HPLC chromatogram of the reaction mixture. The two largest peaks are positioned at the retention times of ouabain and its 1'-epimer. The peak at ouabain's retention time contains the synthetic factor of the invention.

5.2. SYNTHESIS OF OUABAIN PHOSPHATE COMPLEX

Water in ouabain octahydrate (0.5 g, 0.69 mmol) was azeotropically removed with anhydrous pyridine (50°C; repeated 3 times), and the resulting material was dried *in vacuo* for a week. The dried ouabain was dissolved in a mixture of pyridine(0.5 ml)/CH₂Cl₂(10 ml)/THF(15 ml)/DMF(2.5 ml), to which was added MS4A. The mixture was cooled to -78°C. To this solution was added phosphorus oxychloride (10% (v/v) in THF, 2.5 ml, 2 eq.) dropwise over a 15 minute period. The solution turned into white suspension while it was stirred at -78°C for one hour. The mixture was warmed to 0°C over one hour, after which the reaction was quenched with ice-water. The reaction products were extracted with water and evaporated at 50°C to remove residual volatile solvents. The resulting crude solution was dried at 80°C *in vacuo*. The dried material was purified by silica gel column chromatography (20% MeOH in CHCl₃). The mixture of ouabain 1,5,19- and 1,11,19-phosphates, which appeared as a single spot on TLC (R_f=0.35, 40% MeOH in CHCl₃), was collected and concentrated (168 mg; 40% yield as a mixture). A large amount of ouabain (287 mg) was recovered from the reaction.

The mixture of 1,5,19- and 1,11,19-phosphates was further purified by reverse phase HPLC (Waters NovaPak® HR C₁₈, 7.8 x 300 mm, 60 Å, 6 µm; a linear gradient of 0-25% acetonitrile in water over 15 minutes, followed by a 25-50% acetonitrile gradient over 5 minutes; 3 mL/min; observed at 220 nm). For biological studies, purification was repeated to obtain a single peak for each sample.

5.3 CHARACTERIZATION OF SYNTHETIC INHIBITORY FACTORS

A variety of spectroscopic, chemical, and physiological measurements have been performed to characterize specific synthetic inhibitory factors. Although the measurements provided below focus on the ouabain borate and phosphate embodiments of the invention, those skilled in the art will recognize that little or no modification is necessary to characterize the structure and activity of other cardenolide complexes encompassed by the invention.

5.3.1 NMR OF OUABAIN BORATE COMPLEXES

Compounds and complexes of the invention, some of which exhibit biological activity similar to that of HIF, can be distinguished from HIF by spectroscopic techniques such as NMR.

FIG. 4A shows the ^1H -NMR measurement of HIF. This data was obtained by transferring a solution of about 14 μg isolated HIF from bovine hypothalamus dissolved in 10% acetonitrile- d_3 in D_2O (v/v) into a WILMAD 520-1D NMR tube for a Bruker 2.5 mm microprobe. The data was acquired by prolonged accumulation (ns = 10,000).

5 Acetonitrile- d_3 was used as an internal standard.

FIG. 4B shows the ^1H -NMR spectrum of ouabain (10 μg) measured under the same conditions except for the number of accumulation (ns 2,000). Acetonitrile- d_3 was again used as an internal standard.

FIG. 4C shows the NMR ^1H NMR spectrum of ouabain borate. For these
10 measurements, the 20 mg of material was used. This spectrum, which was obtained using the large scale preparation described above, shows the two distinct set of signals with a ratio of approximately 3:2 that is observed in the HIF ^1H NMR spectrum. Since the mass spectroscopy results (described below) show that the ratio between ouabain and borate is 1:1, the two sets of NMR signals are assumed to arise from two stable coordination
15 isomers of ouabain borate.

With reference to the structure shown in FIG. 5, specific chemical shifts and their assignments are shown in Table 2:

Table 2. ^1H and ^{13}C -chemical Shift
Assignment of Ouabain 1,5,19- and 1,11,19-borates

	Ouabain 1,5,19-borate			Ouabain 1,11,19-borate	
	Acetonitrile- d_3 /D $_2$ O(1:1) (310 K)		#	Acetonitrile- d_3 /D $_2$ O(1:1) (310 K)	
	^{13}C	^1H		^{13}C	^1H
5	74.1	4.77	1	76.4	3.46
	33.2	2.05, 1.81	2	39.1	2.00, 1.63
	71.1	3.95	3	71.1	3.56**
	34.6	2.04, 1.59	4	44.1	1.85, 1.62
10	74.2	-	5	75.3	-
	36.4	1.77, 1.42	6	31.1	1.63, 1.40
	24.0	1.81, 1.19	7	22.0	1.80, 1.40
	40.0	1.64	8	36.3	2.20**
15	45.1	1.55	9	52.4	1.43
	41.3	-	10	44.9	-
	67.8	3.62	11	67.2	3.80
	49.7	1.61, 1.38	12	47.7	1.65, 1.42
20	50.7	-	13	50.5	-
	86.0	-	14	87.5	-
	32.3	2.06, 1.67	15	32.8	2.11, 1.71
	27.4	2.12, 1.74	16	27.0	2.14, 1.74
25	50.4	2.81	17	51.1	2.86
	17.0	0.79	18	17.0	0.88
	65.5	4.27, 3.83	19	61.3	3.92
	179.1*	-	20	179.5	-
30	75.8	4.92	21	75.9	4.95
	117.1	5.88	22	117.1	5.91
	179.0*	-	23	179.0*	-
	98.4	4.80	1'	98.8	4.83
35	71.5	3.86	2'	71.4	3.79
	71.1	3.85	3'	71.1	3.67
	73.3	3.30	4'	73.0	3.32
	69.2	3.77	5'	69.6	3.68
	17.5	1.17	6'	17.5	1.19

* Assignments in the same column can be reversed.

** Signal broadening observed.

It is apparent from Table 2 that the most notable structural difference between the two isomers of ouabain borate is seen in the A-ring. The chemical shift of H-1 in the major isomer is 3.46 ppm, while the same proton in the minor isomer appears at 4.77 ppm.

In addition to the ^1H measurements, other NMR techniques (e.g. COSY, HMQC, HMBC, ROESY, ^{13}C , DEPT) were used to determine the resonance assignments of major and minor components of ouabain borate. For these measurements, approximately 20 mg of the synthetic inhibitor was dissolved in deuterium oxide (0.5 ml), although measurements of ^1H - and ^{13}C -NMR spectra also used the 10% acetonitrile- d_3 in D_2O (v/v). Each sample solution was transferred into a 5 mm NMR tube, and measured with a 5 mm probe. HDO, or the methyl groups of acetonitrile- d_3 , where appropriate, were used as internal standards. For ^{11}B NMR measurements, boron trifluoride diethyl etherate was used as an external standard. ROESY spectra were measured with a spin-lock time of 100 ms.

ROESY spectra of the ouabain borate complex show a 100 ms crosspeak between H-1 and H-9 of the major isomer. This suggests that the A-ring of the major component is distorted into a boat-like form, in which H-1 is placed at a shielded axial-like position and sterically closer to H-9. In addition, the negative ROESY crosspeaks that are clearly seen between H-1(major) and H-1(minor), and between H-18(major) and H-18(minor), indicating the structural exchange between the two coordination isomers. During high temperature measurements aimed at examining this structural exchange, signals were observed to broaden as the temperature was raised, but did not coalesce at the highest temperature studied (350° K).

The ^{11}B -NMR measurements of ouabain borate, in combination with the MS/MS fragmentation analysis described herein, provided information regarding the boron coordination site. MS/MS analysis of the ouabain borate ion at 633.24 (ouabain borate with sodium) gave a dominant fragment ion at 487.24, corresponding to ouabagenin borate with sodium. Therefore, borate is strongly attached to the steroid backbone portion of ouabain. Furthermore, 96 MHz ^{11}B -NMR showed a borate peak at 0.78 ppm (broad singlet) from $\text{BF}_3\cdot\text{OEt}_2$, which indicates that the borate is tetrahedrally coordinated. Kidd, R. G., NMR of Newly Accessible Nuclei (Laszlo, P., ed.) pp. 49-77 (Academic Press, New York).

The ^{11}B -NMR signals of the two isomers, which are supposedly close to each other (the borate ester chemical shift is not very sensitive to its substitution), were not distinguished, probably because of the short T_1 relaxation time of the ^{11}B nucleus ($I = 3/2$). For the same reason, long range couplings ($^2J_{\text{BC}}$, $^3J_{\text{BH}}$) were not observed.

Although the invention is not limited by theory, the conclusion drawn from this data is that the ouabain borate has two borate coordination isomers, as shown in FIG. 6.

As shown, the MS/MS and ^{11}B -NMR results suggest that tetrahedral borate is attached to the steroid backbone portion of ouabain. The sites that can reasonably

accommodate the tetrahedral borate group are 1,11,19- and 1,15,19-hydroxyl groups. The major isomer observed in the NMR analysis fits the 1,11,19-borate, in which the coordinated borate stabilizes the A-ring boat conformation. On the other hand, ROESY crosspeaks in the minor isomer are consistent with the 1,5,19-borate, in which the borate formation fixes the A-ring into boat conformation.

5.3.2 NMR OF OUABAIN PHOSPHATE COMPLEXES

Like the borate complexes of the invention, the phosphate complexes of ouabain were also characterized by NMR. Analysis of this data was facilitated by MALDI TOF MS measurements of the complexes.

MALDI TOF MS of ouabain 1,5,19-phosphate gave signals at m/z 650.704 ($M+Na$)⁺, and 666.714 ($M+K$)⁺, whereas signals at m/z 628.73 (M)⁺, 650.773 ($M+Na$)⁺ and 666.758 ($M+K$)⁺ were obtained from the 1,11,19-phosphate. MS spectra confirmed that the phosphate group in each isomer was attached to ouabain through its three hydroxyl groups.

¹³C-NMR of ouabain 1,5,19-phosphate showed two bond $P-C$ couplings ($^2J_{PC}$) at the 1-C (86.5 ppm, $J = 5.7$ Hz), 5-C (89.7 ppm, $J = 4.6$ Hz), and 19-C (73.4 ppm, $J = 4.6$ Hz) signals. Distinct down field shifts of proton and carbon signals at the 1-, 5-, and 19-positions further confirmed the site of phosphorylation. COSY spectra showed a crosspeak arising from a through-space coupling between $7_{ax}-H$ and 15_b-H , which indicates that the molecular conformation is rigidly fixed by the phosphate attachment. ¹³C-NMR of ouabain 1,11,19-phosphate gave clear $^2J_{PC}$ couplings at 1-C (85.3 ppm, $J = 5.7$ Hz) and 19-C (71.0 ppm, $J = 7.0$ Hz), whereas coupling between the phosphate and 11-C was obscured by the broadening of the ¹³C-signal at C-11. The ¹³C-signal broadening also occurred at 2-C, 4-C, 6-C, and 8-C, indicating molecular movement of this isomer. Down field shifts of proton and carbon signals at the 1-, 11-, and 19-positions and NOE between 1-H and 9-H confirmed the phosphate attachment site of this isomer.

With reference to the structure shown in FIG. 5, specific chemical shifts and their assignments are shown in Table 3:

Table 3. ^1H and ^{13}C -chemical Shift
Assignment of Ouabain 1,5,19- and 1,11,19-phosphates

5	Ouabain 1,5,19-phosphate			Ouabain 1,11,19-phosphate	
	Acetonitrile- d_3 /D $_2$ O(1:1) (310 K)			MeOH- d_4 (300K)	
	^{31}P -NMR § (δ) - 6.63 ppm (m)			^{31}P -NMR § (δ) - 7.34 ppm (m)	
	^{13}C	^1H	#	^{13}C	^1H
	86.5	5.88	1	85.3	4.35
	32.1	2.28 (2H)	2	38.3**	2.45, 1.94
	71.0	4.09	3	70.7	3.70
10	34.8	2.35 - 2.03	4	44.5**	1.96, 1.78
	89.7	-	5	74.1	-
	32.5	2.07, 1.68	6	32.2**	1.71, 1.55
	24.0	2.04, 1.29	7	22.3	2.10, 1.59
	39.7	1.69	8	36.9**	2.54
15	46.0	1.72	9	52.3	2.05
	42.0	-	10	46.3	-
	67.2	3.60	11	75.8**	4.59
	49.4	1.65, 1.40	12	46.0	1.98, 1.72
	50.6	-	13	50.8	-
20	85.1	-	14	86.1	-
	34.8	2.11, 1.82	15	33.6	2.20, 1.84
	27.4	2.09, 1.77	16	27.4	2.21, 1.88
	50.5	2.82	17	51.7	2.96
	17.0	0.85	18	16.9	16.9
25	73.4	5.11, 4.68	19	71.0	4.82 (2H)
	177.8*	-	20	177.0	-
	75.3	4.90	21	75.3	4.98
	117.6	5.85	22	118.5	5.95
	177.6*	-	23	177.1*	-
30	99.9	4.77	1'	100.5	4.83
	71.8	3.76	2'	72.6	3.76
	71.4	3.65	3'	72.4	3.64
	73.1	3.33	4'	74.0	3.37
	69.6	3.72	5'	70.3	3.65
35	17.7	1.20	6'	18.0	1.26

* Assignments in the same column can be reversed.

** Signal broadening observed.

\S ^{31}P -NMR external reference is 85% H_3PO_4 .

5.3.3 HPLC OF OUABAIN BORATE COMPLEX

Characterization of the inhibitory factors of the invention is naturally hampered by the presence of impurities. Such impurities are almost always removed by those skilled in the art by HPLC. HPLC is also a useful analytical technique universally employed in the development and investigation of cardiac glycosides and related steroid complexes.

After a great deal of effort, however, the present inventors have found that reverse phase HPLC of some of the synthetic inhibitors of this invention is highly erratic and poorly reproducible. This is attributed to decomposition of the complexes on the column. The apparent decomposition of ouabain borate, for example, may be due to the same mechanism that leads to its decomposition under acidic conditions.

HPLC analysis of ouabain borate was performed on a Waters HPLC system equipped with a 600E solvent delivery system and a 996 photodiode array detector. Data were obtained and processed with a Millennium Chromatography Manager V2.10. Samples were analyzed on a Vydac 218TP (C₁₈, 4.6 x 250 mm, 300 Å, 5 µm) column with a linear gradient of acetonitrile (5-25% over 20 minutes) at a flow rate of 1 mL/min. UV absorption was monitored between 200 nm and 300 nm and at 220 nm.

The instability of ouabain borate is apparent when a small sample (e.g., less than about 5 µg) is injected into a typical analytical column (Vydac 218TP: C₁₈, 4.6 x 250 mm, 300 Å, 5 µm). The sample appears to decompose to reform ouabain, resulting in a retention time equal to that of ouabain. But further complicating the analysis is the fact that upon collection of the peak in a borosilicate test tube or vial, the synthetic factor can reform.

It has also been found that the addition of 0.1% TFA to the HPLC eluent completely converts much larger amounts of ouabain borate to ouabain. Consequently, mixed results are obtained when larger samples are used, but the eluent does not contain TFA or is not acidic.

5.3.4 HPLC AND CD OF NAPHTHOYLATED INHIBITORS

Pentanaphthoylated derivatives of some of the synthetic inhibitors of this invention have been synthesized to assist in their characterization. Naphthoyl derivatives were made because are generally obtained in high yield, give rise to fluorescent products, exhibit intense UV absorptions, and exhibit characteristic exciton-coupled CD spectra (Ikemoto, N., et al. Angew. Chem. Int. Ed. Engl. 31:890-891 (1992)).

For example, ouabain borate was obtained by dissolving approximately 300 ng ouabain in about 100 µl water, and placing the solution in a 1 dram borosilicate glass vial. The sample was kept at room temperature for 3 hours to assure the formation the ouabain borate, and lyophilized. Naphthoylation was performed by adding to the solution 1-(2-naphthoyl)-imidazole (2 mg in 200 µl anhydrous acetonitrile) and 1,8-diazabicyclo-

[5.4.0]undec-7-ene (20 μ l of 2% anhydrous acetonitrile solution). The resulting mixture was stirred at room temperature for 2 hours and then quenched with water (0.5 mL).

Acetonitrile was removed under reduced pressure, and the resulting white suspension was loaded onto a Waters C₁₈ SepPak cartridge. The cartridge was washed with 20%, 40%, and 50% acetonitrile in water (10 ml, 10 ml, and 5 ml, respectively). The naphthoylated products were eluted with acetonitrile (5 ml) and subjected to a Vydac 218TP (C₁₈, 4.6 x 250 mm, 300 Å, 5 μ m) with an isocratic elution of acetonitrile/water (82:18) at 1 mL/min. The chromatogram was monitored by a Shimadzu RF-551 fluorescence detector (λ_{ex} = 234 nm, λ_{em} = 360 nm).

FIG. 7A shows the chromatogram of naphthoylated ouabain borate. FIG. 7B shows that of ouabain pentanaphthoate under the same conditions. Under these conditions, the naphthoylated derivative of the synthetic inhibitor has a shorter retention time than does ouabain pentanaphthoate. This is confirmed by co-injection of the two samples, as shown in FIG. 7C.

Even more significant, the HPLC retention time of naphthoylated ouabain borate appears to be the same as that of naphthoylated HIF obtained using the same naphthoylation protocol. Similarly, the circular dichroism (CD) spectrum of the naphthoyle derivative of the synthetic factor, like the naphthoyle derivatives of HIF, showed no distinct CD curve. FIG. 8 shows the CD spectrum of naphthoyle derivative ouabain borate. The CD spectrum was measured in acetonitrile with JASCO J-720 spectropolarimeter. Parameters for CD measurement were as follows: Bandwidth 1.0 nm; Slit width Auto; Sensitivity 10 mdeg; Response 4s; Start wavelength 400 nm; End wavelength 200 nm; Scan speed 100 nm/min; Step resolution 0.5 nm; Accumulation 4.

The UV spectrum of the "zero-CD" product clearly shows it is a naphthoate derivative, and the reversed phase HPLC retention time indicates that the product is fairly hydrophobic. However, FABMS measurement of the derivative gave only several weak peaks in the low molecular weight region, indicating that the product is not the naphthoate of ouabain borate but rather some smaller (or possibly unstable) byproduct.

5.3.5 ELECTRON SPRAY MS OF OUABAIN BORATE COMPLEX

Ouabain borate was characterized by electrospray MS (ESP) analysis. Although samples prepared as described herein did not give a clear-cut result on FABMS measurements (glycerol matrix), electrospray MS analysis, in which neutral buffer was employed, showed a distinct peak of ouabain borate with sodium at 633.2 together with a smaller ¹⁰B isotope peak at 632.26. Some part of the sample decomposed back to ouabain during this electrospray MS analysis, but the complexation between ouabain and borate is strong enough to be observed under neutral pH conditions.

FIG. 9 shows the MS analysis of this embodiment of the synthetic factor. The exact mass of 632.26 is equivalent to a sodium salt of either a trigonal or tetrahedral borate complex of ouabain having the chemical formula $C_{29}H_{42}BNaO_{13}$.

FIG. 10 shows the MS/MS fragmentation pattern of the synthetic factor, which provides evidence regarding the location of the boron coordination site.

5.3.6 BIOLOGICAL ASSAYS

The biological and/or pharmacological effect of the synthetic inhibitory factors of this invention can be determined by methods known to those skilled in the art. These include, but are not limited to, measurement of the factors' effect on cultured cardiac cells' $^{86}Rb^+$ uptake, cytosolic free Ca^{2+} ($[Ca^{2+}]_i$), and contractility. See U.S. Patent No. 5,716,937, which is incorporated herein by reference.

Although other cells can be used, it is preferred that each of these methods employ myocardial cells isolated from ventricle fragments of rats by serial trypsinizations in a Ca^{2+} and Mg^{2+} -free Hanks Buffered Salt Solution as described by Yagev et al. (Yagev, S., et al. *In Vitro* 20:893-898 (1984)). The trypsinized cells are then decanted into HamF10 medium containing 20% serum and antibiotics and centrifuged at 1000 r.p.m. for 10 minutes. The resulting cell pellet is then re-suspended in HamF10 medium containing fetal calf serum and 10% horse serum with 0.1% penicillin-streptomycin, and diluted to a concentration of 5×10^5 cells/mL. Incubation of the resulting culture for about three days in humidified 5% CO_2 , 95% air at $37^\circ C$ typically yields confluent monolayers in which about 80% of cells exhibit spontaneous synchronous contractions. At this point, experimental measurements can be performed.

The effect of the inhibitory factors of this invention on Na^+/K^+ -ATPase activity can be estimated in cultured cardiac cells as the difference of $^{86}Rb^+$ uptake observed in the presence and absence of 5 mM inhibitory factor, following the method of Panet et al. (Panet, R., et al. *J. Memb. Biol.* 70:165-169 (1982)). To ensure saturation binding, the myocytes can be pre-incubated with inhibitory factor for about 20 minutes prior to addition of $^{86}Rb^+$. Myocyte monolayers can then be washed with HEPES buffer solution (final concentrations, mM: NaCl 150, RbCl 5, HEPES-Tris 10 (Ph 7.0), $CaCl_2$ 1, $MgCl_2$ 5, glucose 10), and the uptake initiated by covering the myocytes with 0.5 mL of the same solution pre-warmed to $37^\circ C$ and containing 2 μCi $^{86}RbCl$. Incubations are then continued for a period during which the uptake of the isotope is linear. This is typically 10 minutes (Heller, M., et al. *Biochem. Biophys. Acta* 939:595-602 (1988)). The uptake can then be determined by aspiration of the reaction mixture followed by two rapid rinses with 3 mL ice-cold $MgCl_2$ (125 mM) and two with 5 mL ice-cold NaCl (165 mM). The cells are then

lysed with 0.6 mL of 0.1N NaOH containing 0.1% (w/v) sodium dodecylsulfate, and the radioactivity counted in a scintillation medium such as Instagel (Packard).

The effect of the inhibitory factors of this invention on Na^+/K^+ -ATPase activity can also be estimated in cultured cardiac cells by measuring changes in $[\text{Ca}^{2+}]_i$ using the fluorescent probe fura-2 (Grynkiewicz, G., et al. *J. Biol. Chem.* 260:3440-3450 (1985)). The theoretical basis of this experiment is discussed in the literature (see, e.g., Tsien, R.W., and Carpenter, B.U. *Fed. Proc. Am. Soc. Exp. Biol.* 37:2127-2131 (1978)).

In this method, myocytes are placed in buffered salt solution (BSS, containing in mM: NaCl 140, KCl 5, CaCl_2 1, MgCl_2 1, glucose 10, Na_2HPO_4 1, HEPES-Tris 10 (Ph 7.4)) to which 5 μM fura-2/AM is added, and incubated for 1 hour in humidified 5% CO_2 -95% air at 37° C. Additional loading medium is added and incubation continued for 15 minutes to complete the hydrolysis of fura-2/AM. The cells are then washed, incubated an additional 30 minutes in BSS, and inserted into a thermostated (37° C) cuvette containing BSS and desired amounts of synthetic factor. The fluorescence is then continuously recorded by a spectrofluorometer. It is preferred that the spectrofluorometer alternate rapidly (e.g., 60 Hz) between dual excitation wavelengths of 340 nm and 380 nm. Emission at 505 nm is measured. Values of $[\text{Ca}^{2+}]_i$ are calculated from the ratio $R = F_{340}/F_{380}$ using the formula $[\text{Ca}^{2+}]_i = K_d B(R - R_{\min}) / (R_{\max} - R)$, where K_d is 225 nM. R_{\max} and R_{\min} must be determined in separate experiments using digitonin to equilibrate $[\text{Ca}^{2+}]_i$ with ambient $[\text{Ca}^{2+}]$ (R_{\max}), and addition of MgCl_2 (0.1 mM) and EGTA (1 mM) (R_{\min}). For an accurate determination of $[\text{Ca}^{2+}]_i$, background auto-fluorescence must be measured in unloaded cells and subtracted from all measurements.

A third method by which the biological activities of the inhibitory factors of this invention can be measured is the measurement of the factors' effect upon myocyte contractility.

Contractility, determined as amplitude of systolic cell motion and beating frequency, can be measured in individual cells using a phase contrast microscope according to the method of Barry, et al. (Barry, W.H., et al. *Circ. Res.* 56:231-241 (1985)). According to this method, a glass coverslip with attached myocytes is placed in a chamber provided with inlet and exit ports for medium perfusion. The chamber is enclosed in a Lucite box maintained at a temperature of 37° C and placed on the stage of an inverted phase contrast microscope. The cells are then covered with 1 mL medium containing the desired inhibitory factor. During continuous perfusion, medium bathing a cell in the center of a coverslip is preferably exchanged with a time constant of approximately 15 seconds at a flow rate of 0.96 mL/min. The image of the cell is magnified using a 40x objective, and one raster line of it is monitored by the motion detector. New position data should be acquired every 16 msec or faster for an image border of a microsphere within the cell layer

moving along the-raster line. The analog voltage of the motion detector is passed through a low pass filter and calibrated to indicate actual amounts of motion in μm . The derivative of the voltage can be determined electronically and recorded as velocity of motion in $\mu\text{m}/\text{sec}$. The rate, amplitude, and velocity of contractions in the control perfusions should remain stable for several hours prior to the addition of the inhibitory factor. The change in contractility induced by the factor is calculated in comparison with the contractility of the same cells prior to addition of the factor.

These methods of measuring the biological activity of the synthetic inhibitory factors of this invention can be combined with, or replaced by, any others known to those skilled in the art. These include testing of the factors' effect on the pulse rate and blood pressure of hypertensive rats.

5.3.7 BIOLOGICAL ACTIVITY OF OUABAIN BORATE COMPLEX

Ouabain borate, prepared by the borosilicate glassware method described herein, was submitted to the enzyme assays of Na^+/K^+ -ATPase rat $\alpha 1$ isoform, which is insensitive to ouabain (see, e.g., Lingrel et al., *The Sodium Pump* (Bamberg and Schoner, eds.) pp. 276-286 (1994)). As shown in FIG. 11, ouabain borate showed inhibition at 10^{-5} M on the coupled enzyme assay that measures initial velocity (2 minutes) of the ATPase. By contrast, ouabain showed inhibition at 10^{-5} M.

5.3.8 BIOLOGICAL ACTIVITY OF OUABAIN PHOSPHATE COMPLEX

Ouabain 1,5,19- and 1,11,19- phosphates were examined with Madin-Darby canine kidney (MDCK) cell-based assay with a mycrophysiometer (CytosensorTM), which can detect extracellular acidification rate, an index for the metabolic activity of cells. In this assay, MDCK cells were placed in a cell chamber and perfused in a running medium (Dulbecco's Modified Eagle Medium without sodium bicarbonate, pH 7.4). The running medium was periodically stopped and the accumulation of extracellular acid was monitored by silicon-based light addressable potentiometric sensor (LAPS) coupled with light-emitting diode (LED). See, Hafemin, D. G., Parce, J. W. & McConnell, H. M., *Science* 240:1182-1185 (1988); and Owicki, J. C. et al., *Annu. Rev. Biophys. Biomol. Struct.* 23:87-113 (1994). The measurement of cellular acidification rate in the running medium was carried out every two minutes until metabolic activity in MDCK cells became stable, which normally took approximately one hour. Subsequently, the medium containing the sample of interest was introduced to the cell chamber for a short period (typically 6 mm) and changes in the acidification rate during and after the sample treatment were monitored. Usage of the MDCK cell line made it possible to compare the microphysiometer assay results with those of enzyme inhibition assay using dog kidney Na^+/K^+ -ATPase ($\alpha 1$ isoform).

MDCK cells were cultured in D-MEM containing 10% FBS under 5% CO₂.

Subconfluent cells were rinsed twice with 0.3% EDTA / 0.25 % Trypsin / Hank's balanced salt solution (HBSS) without Ca²⁺ (1 mL:1 mL:8 mL, respectively). A small amount of the 0.3% EDTA /0.25 % Trypsin /HBSS solution was kept in the flask after the second rinsing.

5 After 5-10 minutes, when cells were lifted and became spherical, culturing flasks were shaken gently to completely detach the cells from the bottom of the flask. Cells in the small amount of 0.3% EDTA/0.25 % Trypsin /HBSS were transferred into FBS (1 mL) to quench the trypsin reaction, spinned down at 1000 rpm (25°C), and suspended in the growing medium. Cells were counted with a hemocytometer, and 1 x 10⁵ cells were seeded in each
10 flask for culturing. MDCK cells were split and seeded every other day to keep the cells in the log phase.

MDCK cells were seeded into a plate of capsule cups (12 capsule cups/plate) on the day before the microphysiometer experiment. Cells were suspended at 10⁵ cells/mL in the growing medium. One mL of the cell suspension was pipetted into each capsule cup. Two
15 mL of growth medium was pipetted around the outside of each capsule cup. The seeded plate was placed in the CO₂ incubator (5% CO₂, 37°C) overnight. Samples were dissolved in water at desired concentration for each experiment and lyophilized.

On the day of the microphysiometer experiment, a running medium was prepared from powdered D-MEM (without sodium bicarbonate). Powdered D-MEM was added with
20 11.1 mL of 4M NaCl to keep the osmotic balance between the cytoplasm of MDCK cells and the running medium. The powder was completely dissolved in sterile water (final volume 1 L) and its pH was adjusted to 7.2 by 1 M NaOH. Ten mL of FBS was added to the medium. A part of the medium was used to reconstitute the lyophilized sample. The remaining medium and the sample solution were then sterile filtered: the filtration slightly
25 raises the medium pH to 7.4.

The addition of 1% FBS in the running medium was found necessary to obtain the stable cellular acidification rate from MDCK cell line, as the acidification rate of MDCK tends to decrease in the serum free medium. Although serum free medium is recommended by the manufacturer (Molecular Devices, Sunnyvale, CA) of the microphysiometer, the
30 absence of serum diminishes the metabolic activities in MDCK cells, which eventually leads to serum-deprivation induced apoptosis. Henkel, R. D., VandeBerg, J. L. & Walsh, R. A, Anal. Biochem. 169:312-318 (1998).

Sensor chambers were equilibrated to the pH of the running medium for 20 minutes. The Cytosensor™ was flushed with the running media for at least 10 minutes, during which
35 time the valves were switched every 2-3 minutes. Capsules with cells were assembled and placed on the sensor chambers, which were then mounted on the Cytosensor™ for the measurement of acidification rate. Default protocol for pump cycle / acidification rate

measurement was employed (Pump-on: 0 seconds to 1 minute 20 seconds; pump-off 1 minute 20 seconds to 2 minutes; acidification rate measurement 1 minute 28 seconds to 1 minute 50 seconds). The acidification rate, which was measured every two minutes, was monitored for 30 minutes to 1 hour before the valve switch (background) and sample introduction. Obtained data were processed with Cytosoft® V2.0.1.

As shown in FIG. 12, when a large amount of ouabain (10^{-5} - 10^{-4} M) was applied to the MDCK cells, cellular acidification rate went down to 80% of the original level. This indicates that Na^+/K^+ -ATPase was consuming a large fraction (ca. 20%) of the metabolic activity of the cells. Because the depression in the acidification rate persisted even after the withdrawal of ouabain, ouabain appears to bind tightly to Na^+/K^+ -ATPase.

Subsequently, both ouabain 1,5,19- and 1,11,19-phosphates were examined with the Cytosensor™ assay system to see how the MDCK cells react to these conformationally fixed ouabain analogs. As shown in FIGS. 13A and 13B, a clear decrease (ca. 15 %) in the acidification rate was observed when 10^{-5} M of ouabain 1,5,19-phosphate was added to the MDCK cells. The decreased acidification rate remained low after the withdrawal of the 1,5,19-phosphate, indicating that its mode of binding to Na^+/K^+ -ATPase was similar to that of ouabain. This result also supports a conclusion that the 1,5,19-hydroxyl groups of ouabain are not directly involved in the interaction of ouabain with the Na^+/K^+ -ATPase binding site.

As shown in FIGS. 14A and 14B, the 1,11,19-phosphate caused a clear response, i.e., 7 - 8 % decrease, at 10^{-4} M, and the acidification rate came back to the original level soon after the withdrawal of the sample. The decrease roughly corresponded to 35 - 40% inhibition of Na^+/K^+ -ATPase. The quick recovery in the acidification rate indicated a binding profile of the 1,11,19-phosphate that is different from the 1,5,19-phosphate.

Ouabain 1,5,19- and 1,11,19-phosphates were also tested on dog kidney Na^+/K^+ -ATPase ($\alpha 1$ isoform) assay, in which liberated inorganic phosphate is quantified by a molybdate dye complexation. Henkel, R. D., VandeBerg, J. L. & Walsh, R. A., Anal. Biochem. 169:312-318 (1998). The inhibition profiles of dog Na^+/K^+ -ATPase ($\alpha 1$ isoform) by ouabain 1,5,19- and 1,11,19-phosphates correlated well with the respective the microphysiometer assay results. Ouabain 1,5,19-phosphate inhibited 82.3% (standard deviation, S.D., 4.5%) of the ATPase activity at 10^{-5} M, whereas ouabain 1,11,19-phosphate caused 37.4% (S.D. 7.7%) inhibition at 10^{-4} M. The good correlation between the two assays indicates that the actions of the two phosphate analogs on MDCK cells are mediated by Na^+/K^+ -ATPase.

It is clear from the above that cardenolide complexes of this invention are readily made and can inhibit Na^+/K^+ -ATPase. It should further be clear that the embodiments of the invention described above are intended to be merely exemplary and those skilled in the art

will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. All such equivalents are considered to be within the scope of the invention and are encompassed by the following claims.

- 5 The contents of all references described herein are hereby incorporated by reference.

CLAIMS

What is claimed is:

- 5 1. A process for preparing a synthetic Na^+/K^+ -ATPase inhibitory factor comprising contacting a cardenolide having at least two hydroxy groups with a compound capable of forming ester or ether bonds with said at least two hydroxy groups.
2. The process of claim 1 wherein the cardenolide is ouabagenin.
- 10 3. The process of claim 1 wherein cardenolide is ouabain or a derivative, regioisomer, or stereoisomer thereof.
4. The process of claim 1 wherein the compound capable of forming ester or
15 ether bonds comprises an atom selected from the group consisting of boron, carbon, phosphorous, nitrogen, and sulfur.
5. The process of claim 1 wherein the compound capable of forming ester or
20 ether bonds is; or is formed from, phosphorus oxychloride, borosilicate glass, sodium tetraborate, potassium tetraborate, phenylboric acid, or a derivative of phenylboric acid.
6. A synthetic Na^+/K^+ -ATPase inhibitory factor prepared by the process of claim 1.
- 25 7. A method for converting ouabain into a synthetic Na^+/K^+ -ATPase inhibitory factor distinct from ouabain which comprises:
 (a) dissolving ouabain in an aqueous solvent; and
 (b) contacting the ouabain with a source of one or more boron compounds under
 conditions such that a product mixture is formed.
- 30 8. The method of claim 7 wherein the source of one or more boron compounds is borosilicate glass, sodium borate, or potassium borate.
9. A synthetic Na^+/K^+ -ATPase inhibitory factor prepared by the process of
35 claim 7.

10. A method for converting ouabain into a synthetic Na^+/K^+ -ATPase inhibitory factor distinct from ouabain which comprises:

- (a) dissolving ouabain in an aqueous solvent; and
(b) contacting the ouabain with a source of one or more phosphorous compounds under conditions such that a product mixture is formed.

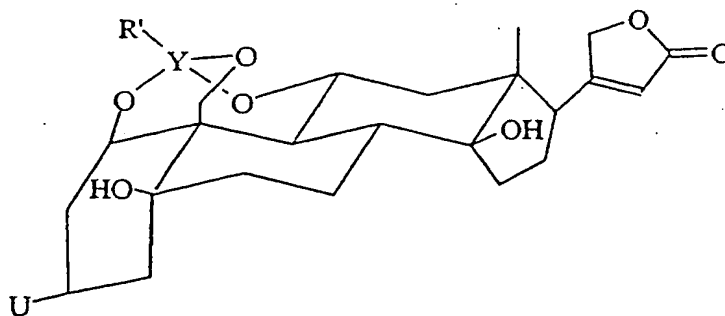
11. The method of claim 10 wherein the source of one or more phosphorous compounds is phosphorus oxychloride.

12. A synthetic Na^+/K^+ -ATPase inhibitory factor prepared by the process of claim 10.

13. A synthetic inhibitory factor that specifically binds Na^+/K^+ -ATPase reversibly and with high affinity, wherein said synthetic inhibitory factor has ^1H and/or ^{13}C nuclear magnetic resonances substantially the same as those listed in Table 2 or 3.

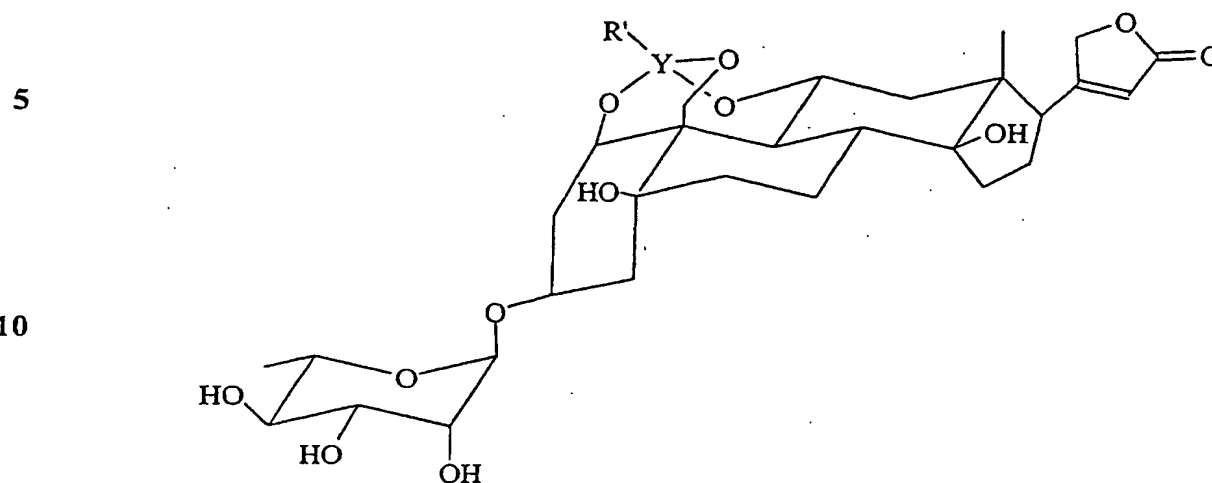
14. A synthetic inhibitory factor that specifically binds Na^+/K^+ -ATPase reversibly with a high affinity, wherein said synthetic inhibitory factor comprises a borate or phosphate complex of ouabain or an isomer or derivative thereof.

15. A compound of Formula 1 or 2:

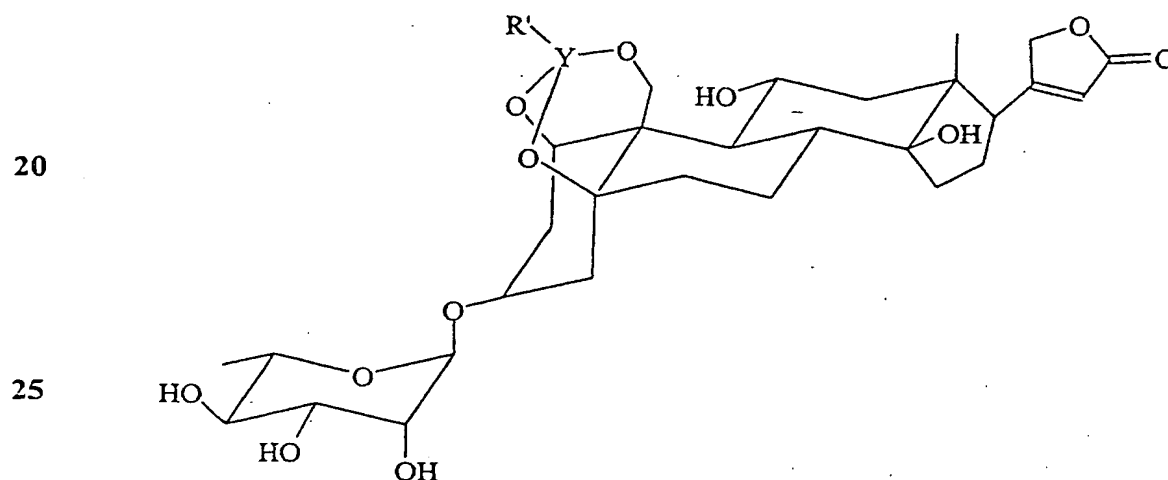


Formula 1

22. A composition comprising amounts of compounds of Formulas 3 and 4:



Formula 3



Formula 4

30 or pharmaceutically acceptable salts, solvates, or clathrates thereof or protected derivatives thereof, wherein each Y is independently boron or phosphorous and each R' is independently oxygen, hydroxy, alkoxy, ester, or phosphate ester.

35 23. A stable 1, 11, 19-substituted ouabain ester having Na⁺/K⁺-ATPase inhibitory activity.

24. A stable 1, 11, 19-substituted ouabain ester having Na^+/K^+ -ATPase inhibitory activity.

5 25. A pharmaceutical composition which comprises a therapeutically effective amount of a compound of claim 6, 9, 12-15, 20, 21, 23, or 24 and a pharmaceutically acceptable carrier.

10 26. A method of preventing or treating cardiovascular disease which comprises administering to a patient in need of such treatment or prevention a therapeutically or prophylactically effective amount of a compound of claim 6, 9, 12-15, 20, 21, 23, or 24.

FIG. 1A

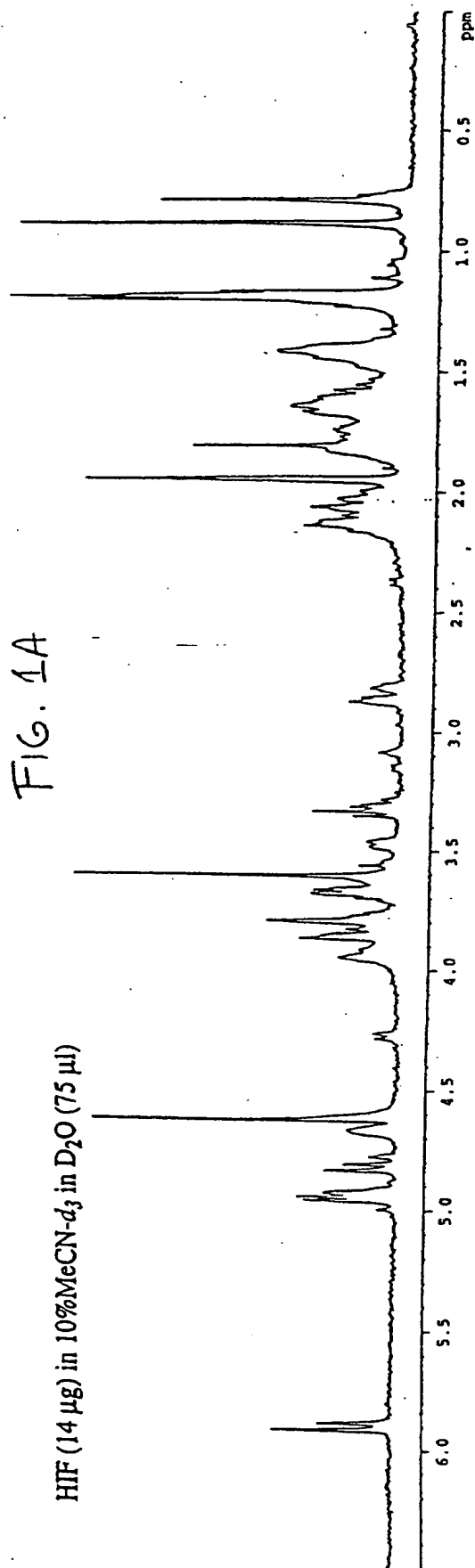
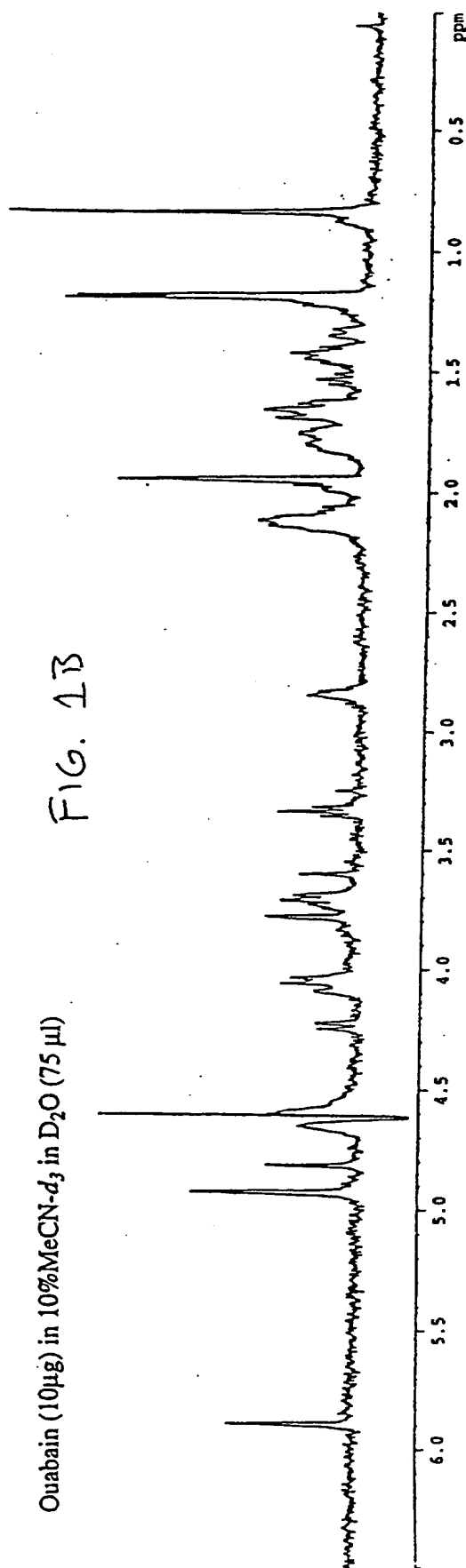
HIF (14 μg) in 10%MeCN- d_3 in D_2O (75 μl)

FIG. 1B

Ouabain (10 μg) in 10%MeCN- d_3 in D_2O (75 μl)

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FIG. 2A

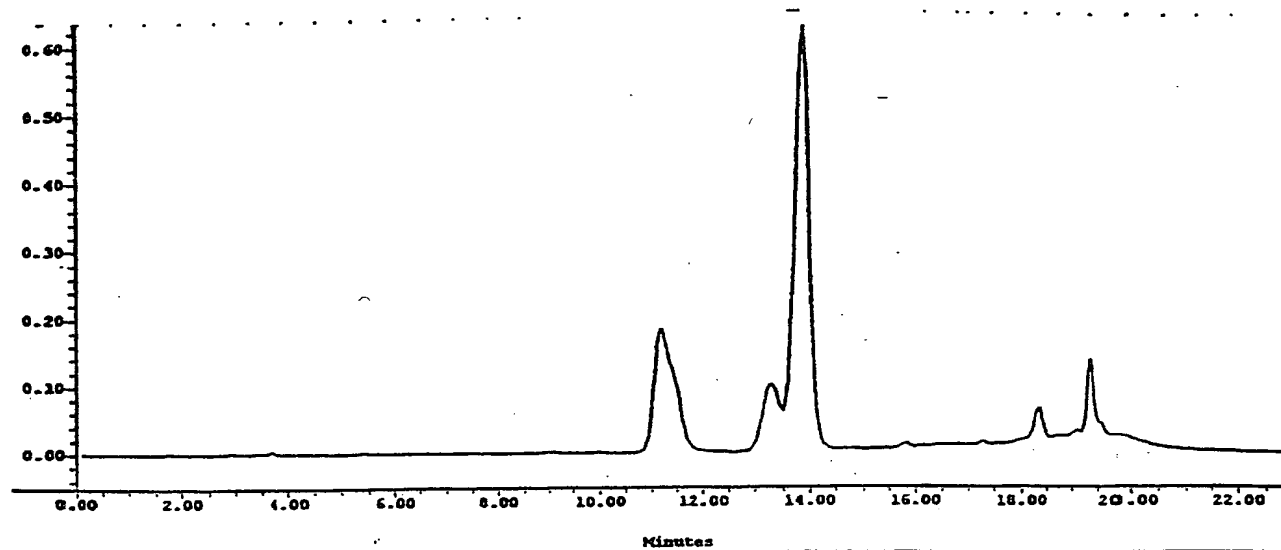
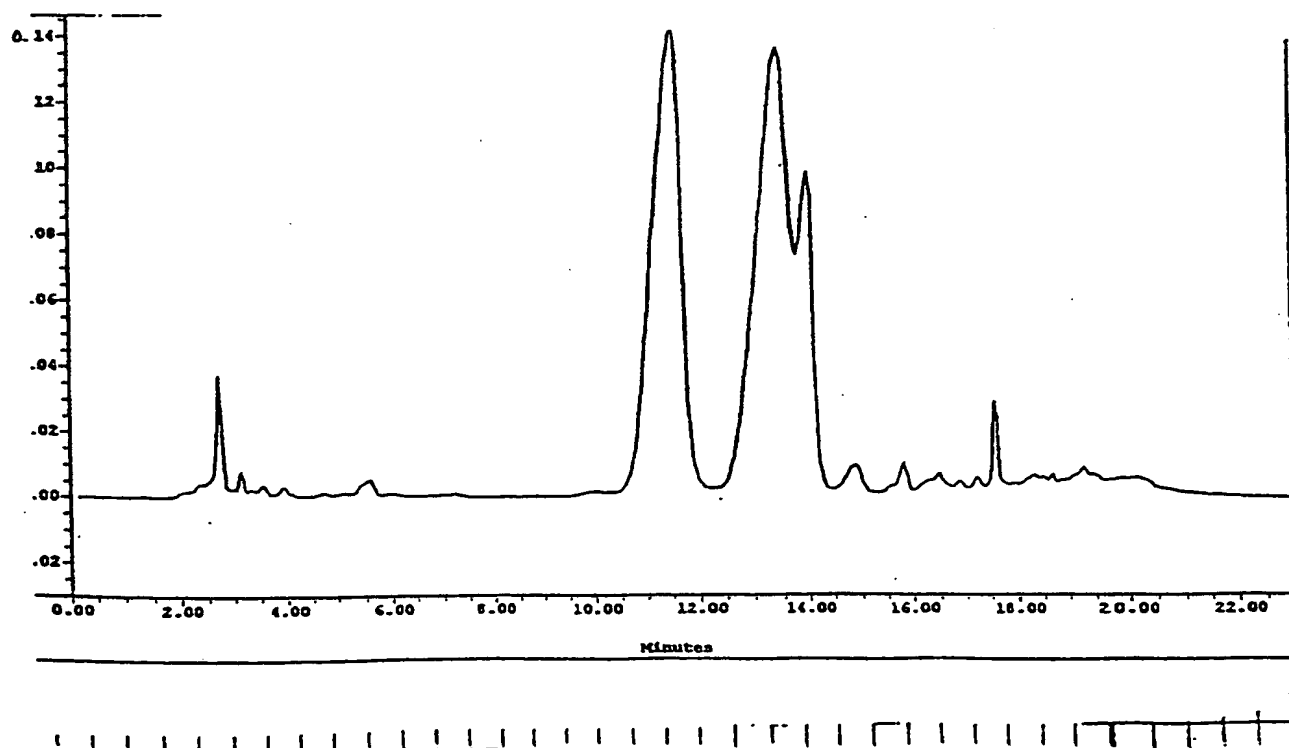
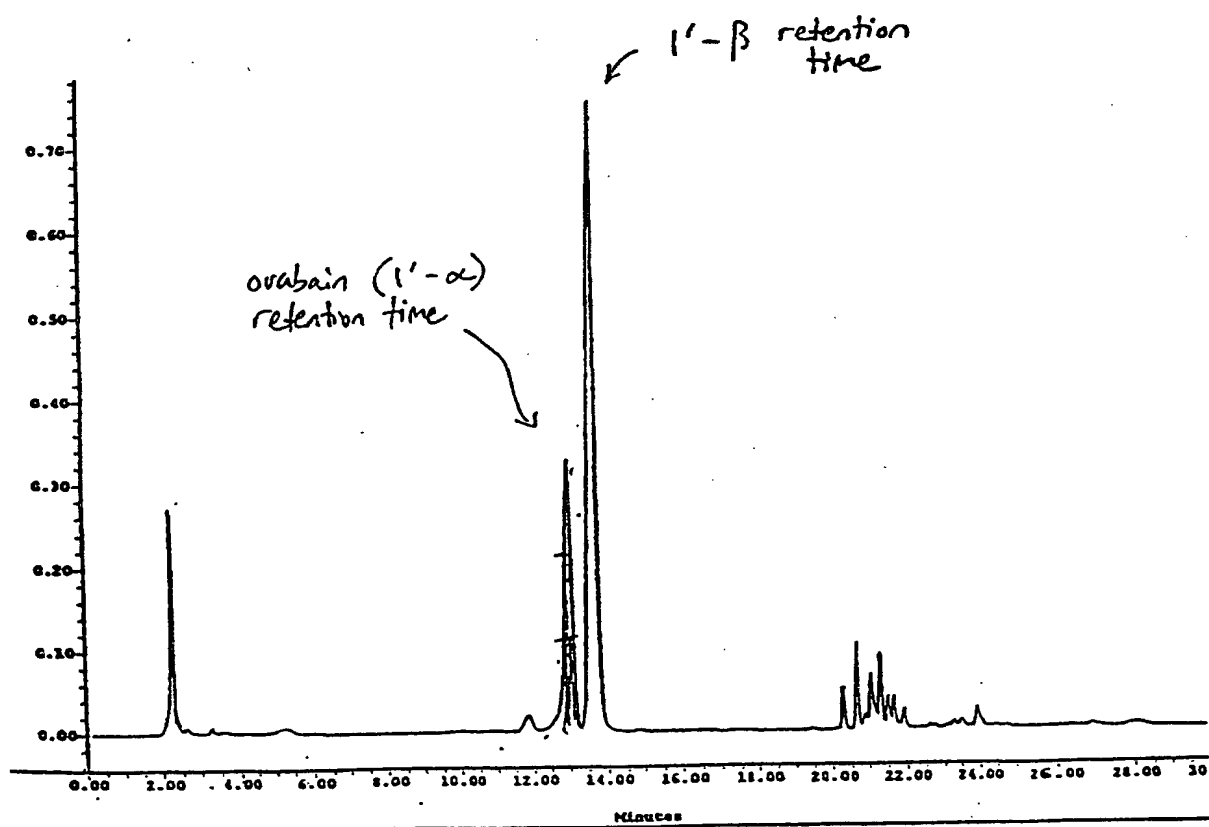


FIG. 2B



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FIG. 3



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FIG. 4A

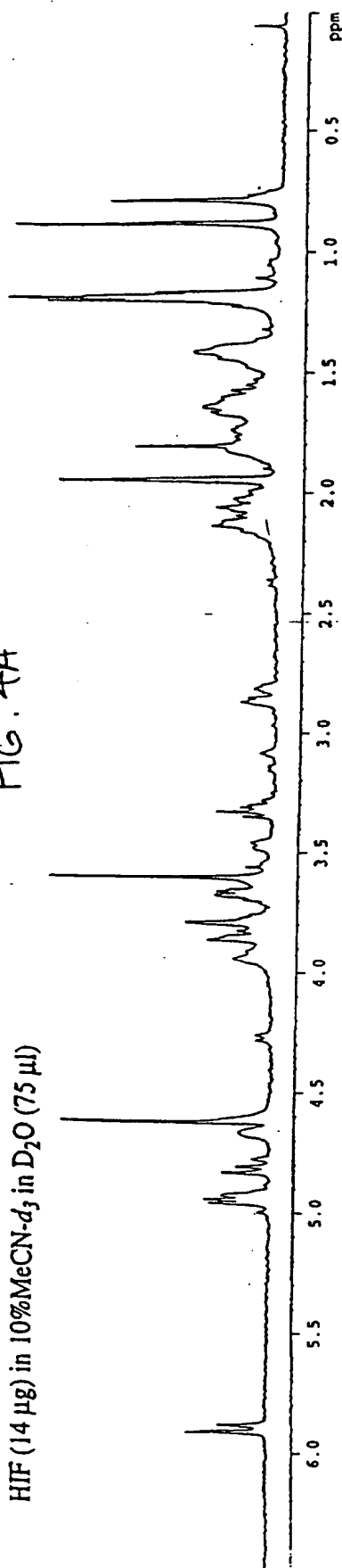
HIF (14 μ g) in 10%MeCN- d_3 in D_2O (75 μ l)

FIG. 4B

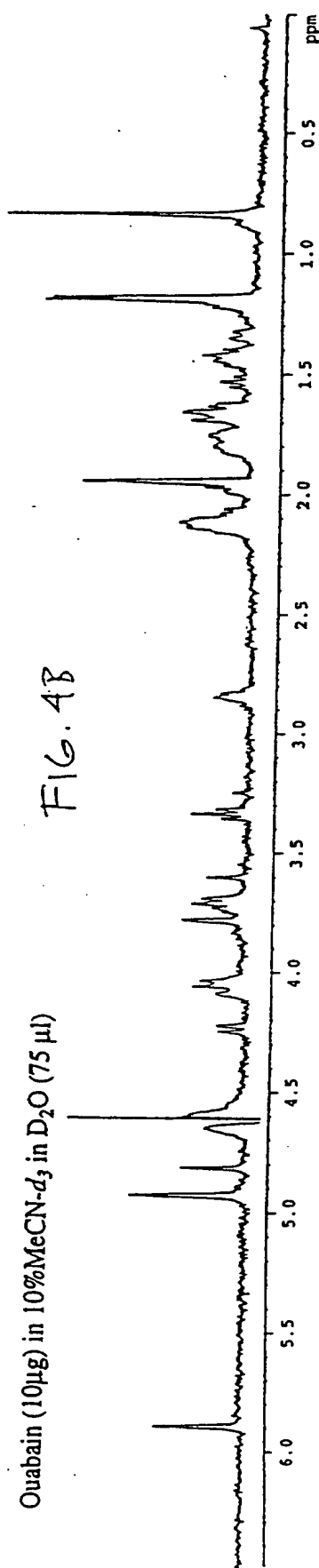
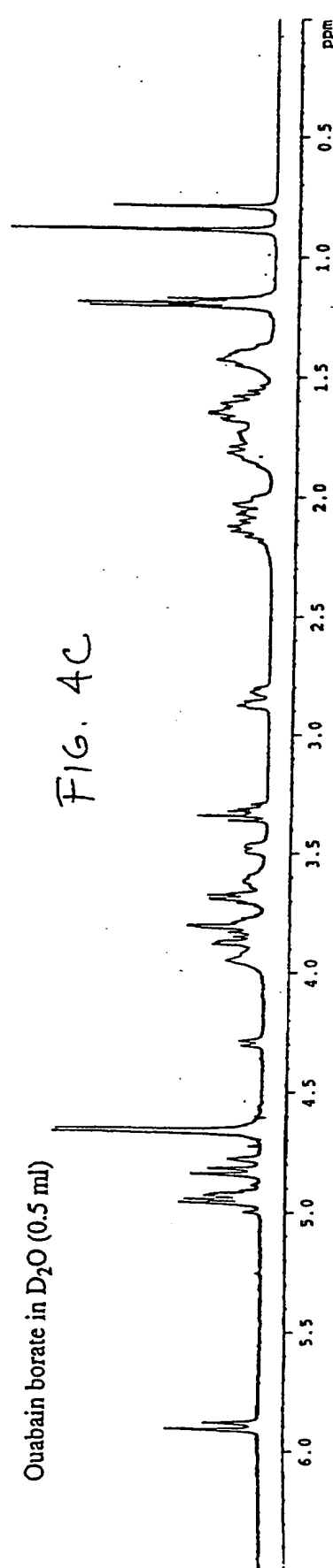
Ouabain (10 μ g) in 10%MeCN- d_3 in D_2O (75 μ l)

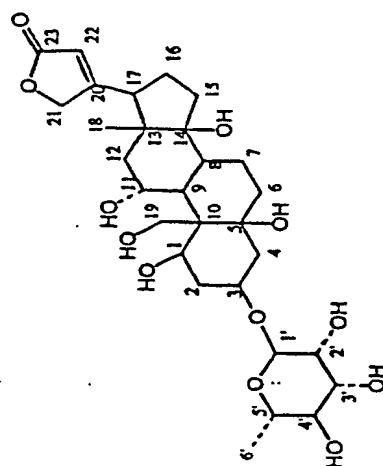
FIG. 4C

Ouabain borate in D_2O (0.5 ml)

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Fig. 5

NMR Assignment of Major and Minor Components
in the Framework of Ouabain Complexes



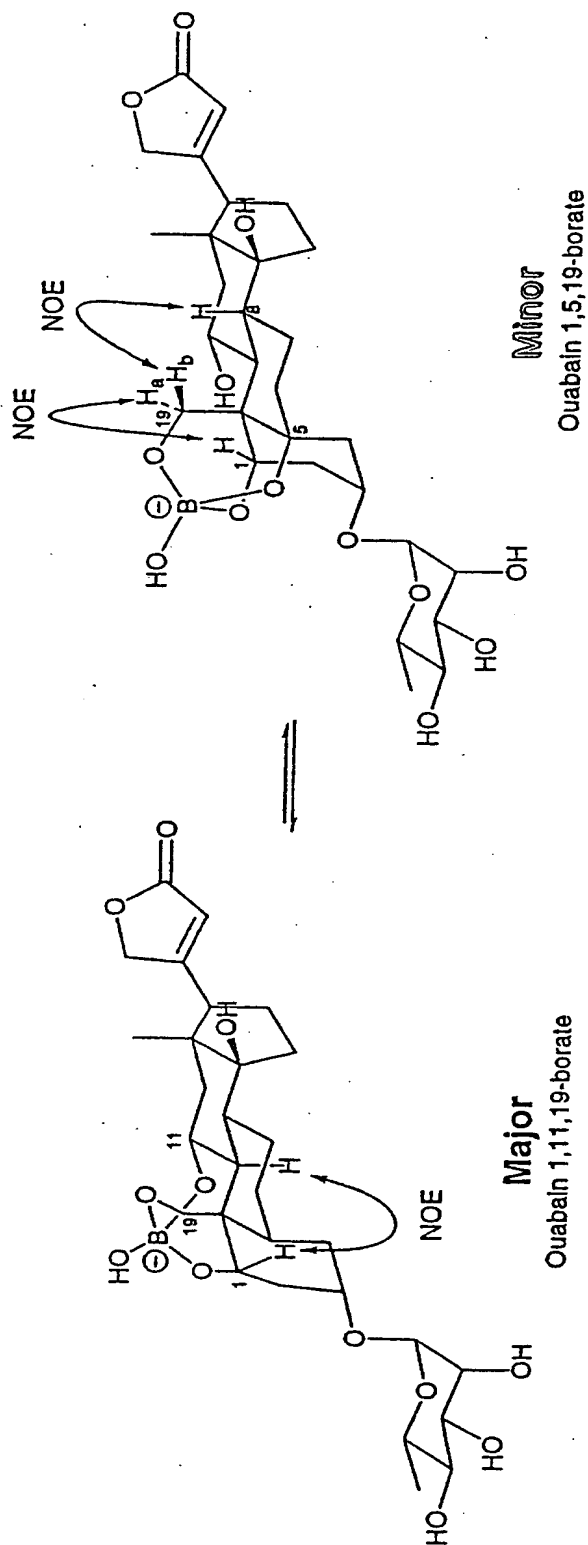


Fig 6

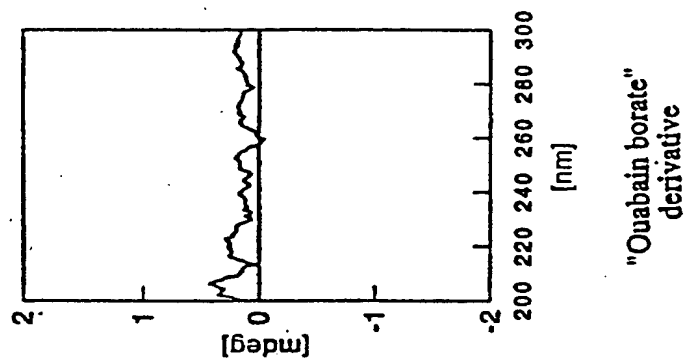
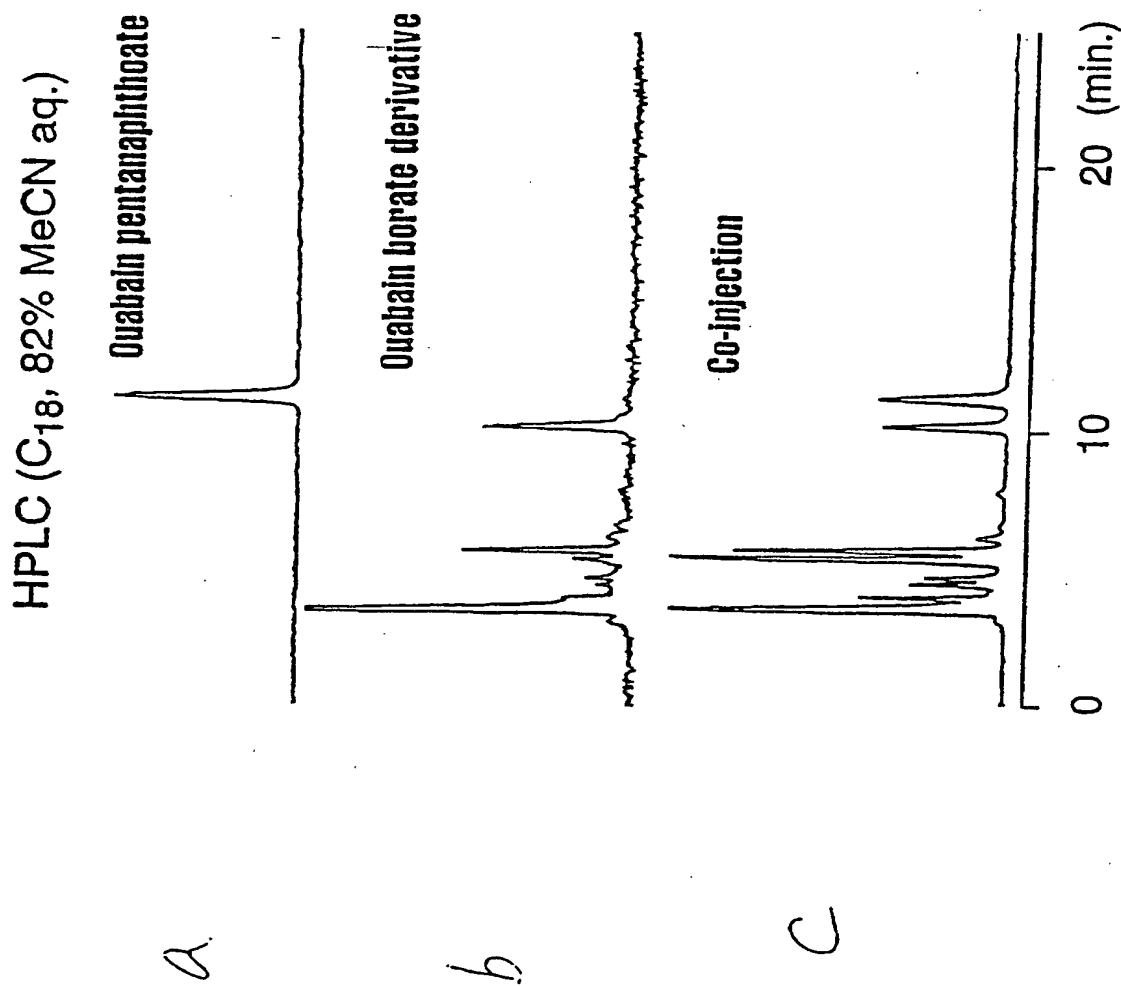
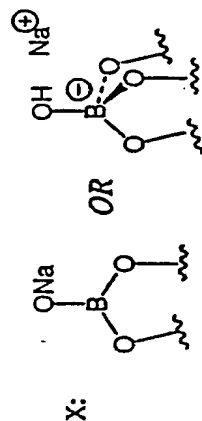
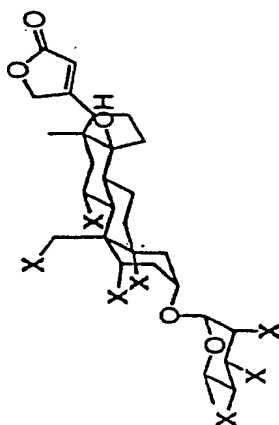
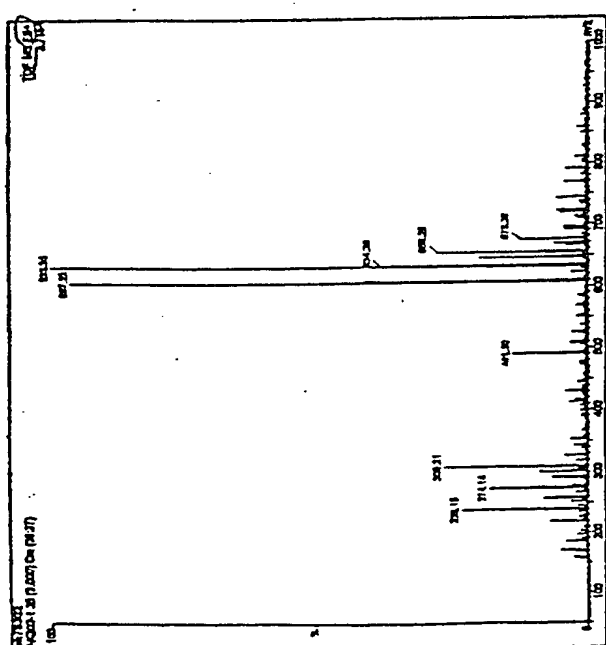
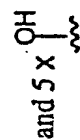


FIG. 8

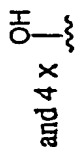
FIG. 7



Trigonal borate
w/ diol



Tetrahedral borate
w/ triol

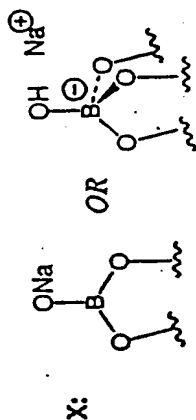
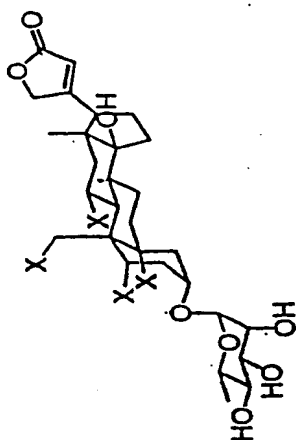


and 5 x OH
and 4 x OH

$C_{29}H_{42}BNaO_{13}$
Exact Mass: 632.26

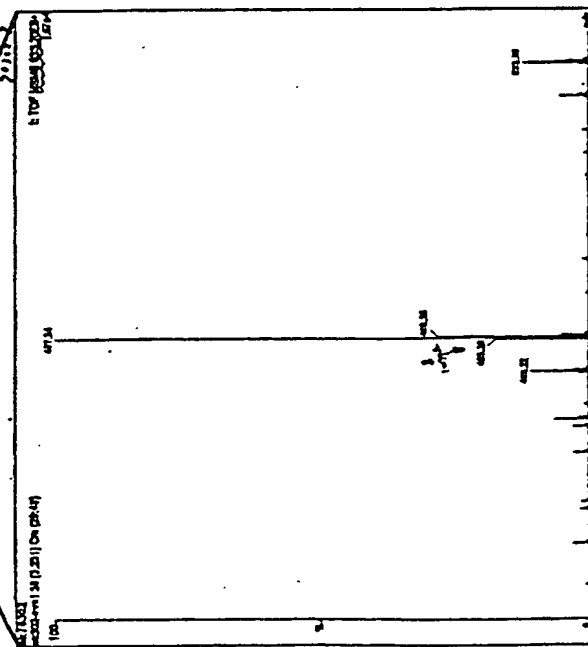
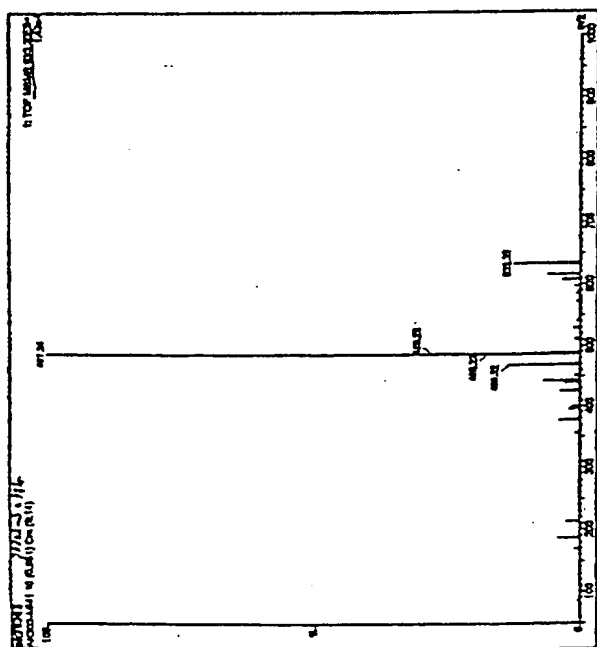
FIG. 9

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$C_{29}H_{42}BNaO_{13}$
Exact Mass: 632.26

FIG. 10



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Inhibition of Na^+, K^+ -ATPase Rat $\alpha 1$ Isoform

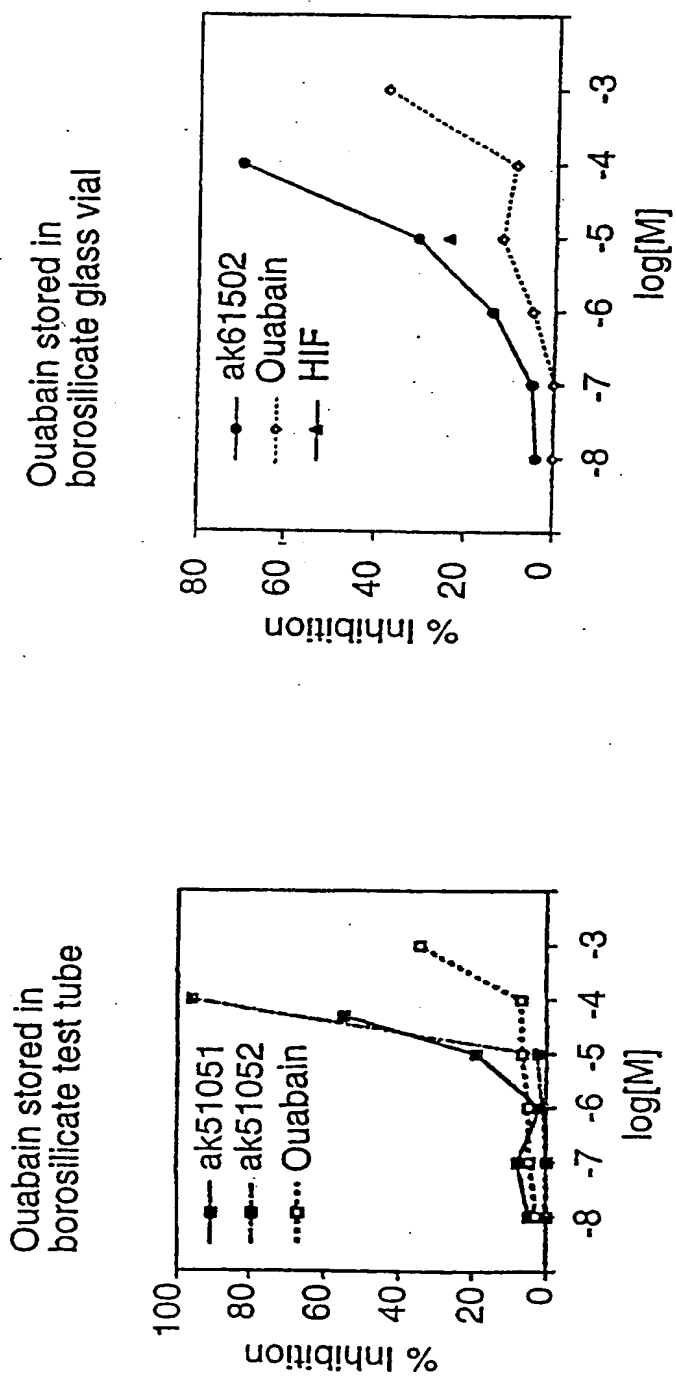


FIG. 11

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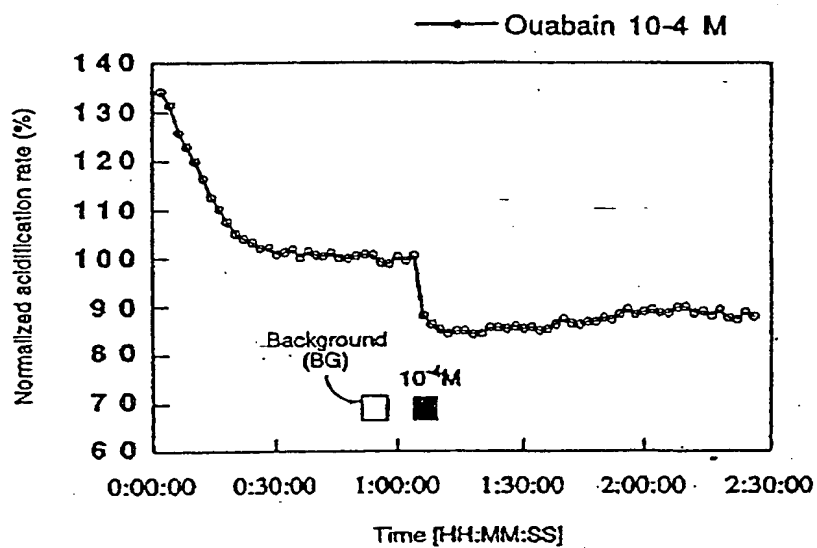


FIG. 12

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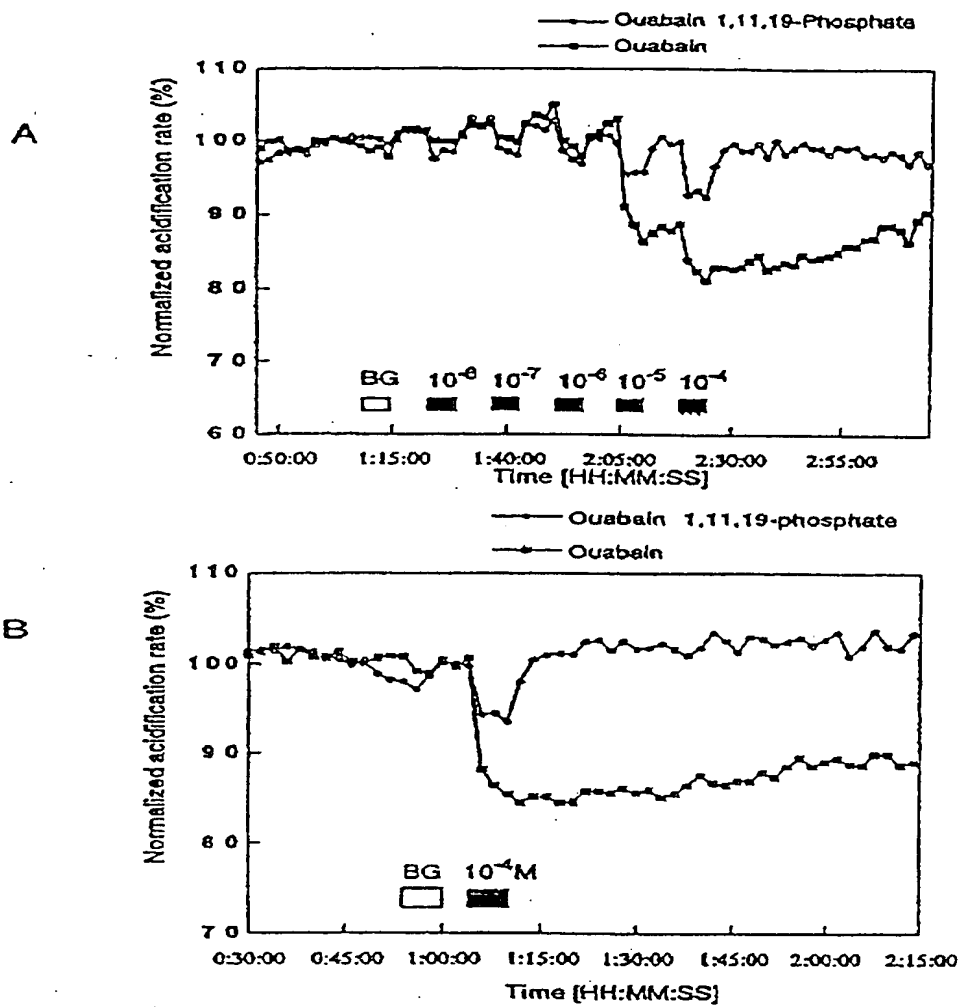


FIG. 13

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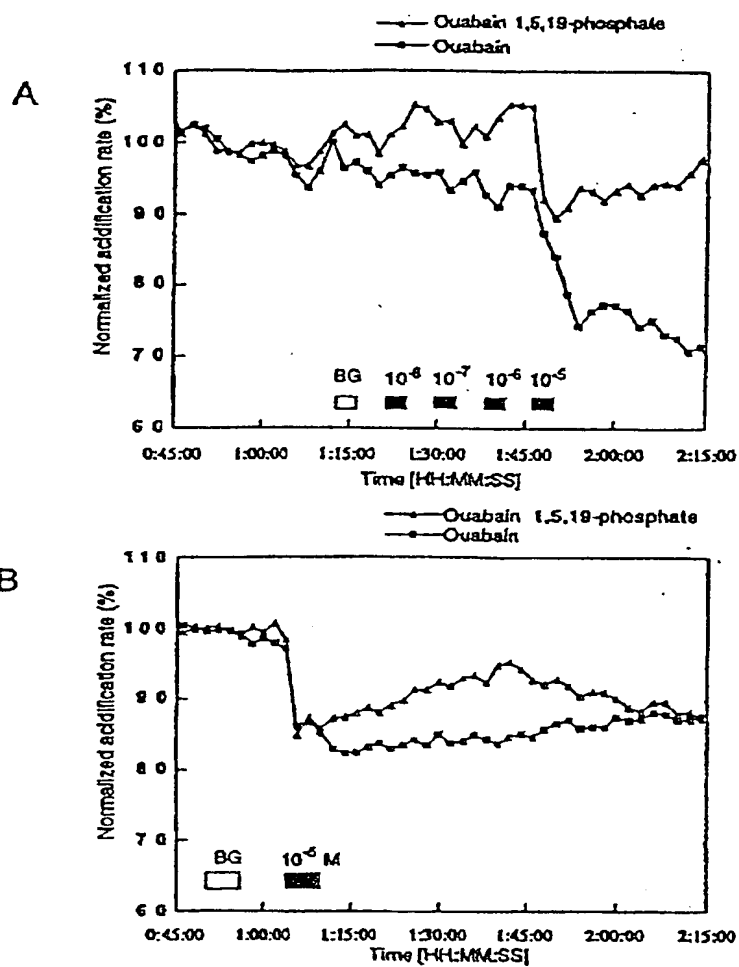


FIG. 14

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